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Truth scarce ever yet carry'd it by Vote anywhere at its first Appearance: New Opinions are always suspected and usually oppos'd, without any other Reason but because they are not already common. But Truth, like Gold, is not the less so, for being newly brought out of the Mine. Tis Trial and Examination must give it Price, and not any antick Fashion:

John Locke. *Essay concerning Human Understanding.* 1690.

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EXPERIMENTAL PATHOLOGY

IS HÆMOLYSED BLOOD TOXIC?

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From the Institute of Physiology, University College, London.

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STATEMENTS have been made from time to time that when hæmoglobin is set free into the plasma it acts as a toxic substance. Further, that the mere defibrination of blood by gentle whipping, which causes the destruction of very few corpuscles, renders its re-injection fatal or very dangerous. Again, the presence in the blood of various substances, such as bile-salts, nitrites, certain aromatic compounds, as aniline or pyrogallol, converts the hæmoglobin into methæmoglobin to a greater or less degree. This change may or may not be accompanied by destruction of the corpuscles with passage of their hæmoglobin or methæmoglobin into solution in the plasma. The serious results of transfusion of blood into a patient whose blood hæmolyses the corpuscles of that introduced are apparently regarded as due to the hæmolysis as such.

Certain experiments which I had occasion to make recently and in which destruction of blood-corpuscles was involved showed an unexpected absence of toxic effects. Further doubt with regard to the truth of the received interpretations was aroused by a letter which I received from Dr. Burkitt, of Nairobi, who had succeeded in the recovery of "hopeless" cases of blackwater fever by the intravenous injection of gum-saline, which was found to raise the blood-pressure and restore the secretion of urine. It is difficult to believe that so inert a substance as gum acacia could have any remedial action other than that due indirectly to the increase of the volume and pressure of the blood.

The question seemed worth some further investigation. We may first consider the results of mere defibrination of the blood. It is well known that the re-injection of defibrinated blood of a rabbit, even into the same animal from which it was taken, is followed by death. This was found to be explained by the production of intravascular clotting. Whether this is due to the exceptional properties of rabbit's blood or to a peculiar propensity of this animal to intravascular clotting is difficult to decide, on account of the toxic properties of blood from a different species in the case of animals in general. Brodie (1) ascribed these toxic properties to a protein in the serum produced in the act of coagulation. It is to be noted, however, that the remarkable properties of foreign proteins, of which anaphylaxis is one manifestation, were unknown at the time of his experiments, only one of which consisted of the injection into a cat of its own defibrinated blood. All the others were with foreign serum. I am inclined to attribute the toxic action obtained in the one case of the defibrinated blood of the cat itself to some accidental circumstance, since I have not met with it in a large number of experiments in which the defibrinated blood of the same or another cat was injected for various purposes. We may conclude that, with the exception of the rabbit, which is peculiarly sensitive to the "fibrin-ferment" produced in clotting, the re-injection of defibrinated blood into the animal itself is harmless. Whether man does or does not behave like the rabbit I am unable to say, not having met with any case in which defibrinated blood has been injected. It appears, however, from some remarks by Quénu (2), that blood-serum has been used in the French Army for the treatment of wound-shock. But no data are given as to the results.

We may pass on to the more interesting problem of hæmolysis.

Couvreur and Clément (3) describe experiments in which they re-injected rabbit's blood, after the corpuscles had been laked, into the same animal from which the blood had been taken. It is unfortunate, for the reason given above, that rabbits were used, and the description of the manner of death—convulsions, with a loud cry—suggests that intravascular clotting had been produced. It is true that one experiment is given in which the serum of clotted blood was innocuous, but it is to be remembered that individual rabbits may be met with in which the phenomenon of intravascular clotting is absent. Mudge (4), indeed, states that albino rabbits are refractory in this respect; but it is doubtful whether his experiments were sufficiently numerous to warrant a general statement.

An experiment made by Paul Bert (5) is given by himself as a proof that hæmoglobin freed from the corpuscles is toxic. A dog was bled until rigidity of the limbs came on. The injection of the blood removed, after it had been frozen and thawed, failed to restore life—in fact, it is stated that death ensued more rapidly than if serum alone had been used. It will be clear that the data are scarcely sufficient to justify the conclusion made. We are not told how long the animal had remained with the very low blood-pressure due to the hæmorrhage, and it is possible that the nerve centres had become irrecoverable. The procedure was too drastic to test the question.

Roger (6) states that, while freshly laked blood is toxic to rabbits, and in doses of 4 to 6·8 c.c. per kilo., it loses this property if allowed to undergo autolysis in an incubator for ten days. These results do not permit any conclusion to be drawn as to whether it was the fibrin-ferment that had disappeared, or whether any other toxic constituent was present in the fresh blood. So far as they go they do not support the view that hæmoglobin itself is toxic. It would probably still be present, although deprived of oxygen, in the autolysed blood, assuming that it remained aseptic.

Barratt (7) also made experiments on the rabbit. He came to the conclusion that the effects of hæmolysed blood were due to intravascular clotting, brought about by the presence in the serum of "thrombokinase," liberated from the stromata of the red cells. He states that he could obtain no evidence of any other toxic constituent.

The suppression of urine in black-water fever has been attributed to the hæmolysis present, but Yorke and Nauss (8) showed that, even in rabbits, hæmolysis alone had not this result, and that it was fall of blood-pressure that was responsible for it. If this happened, the lumen of the renal tubules became clogged with granules of hæmoglobin, doubtless the result of absorption of water from the solution of hæmoglobin filtering through the glomeruli. As the blood-pressure was low this filtrate would be scanty, and in its slow passage along the tubules would lose the greater part of its water by reabsorption. It seems probable that the restoration of renal secretion in Burkitt's cases was due indirectly to the rise in blood-pressure.

My experiments on the question were made with the object of testing whether destruction of red blood-corpuscles was accompanied by the passage of toxic substances into the blood similar to those produced in injured tissues, and having an action similar to that of histamine, as described by Dale and his coadjutors (9, 10). I was unaware at the time of the results of Barratt and of Yorke and Nauss, and therefore somewhat surprised to find that blood hæmolysed by freezing and thawing was not only innocuous to cats, but raised the blood-pressure, if it was low, and increased the secretion of urine. A volume of blood amounting to about one-third of that calculated as being present was first withdrawn, and replaced by an equal volume of gum-saline in order to keep up a normal blood-pressure. The blood was defibrinated by whipping, frozen at − 15°, and thawed. It appeared to be completely hæmolysed. Sometimes there is a second feeble coagulation on thawing, which makes it advisable to whip the blood gently with a feather during the thawing. Straining through muslin is sufficient to remove small clots, etc. The stromata, not being disintegrated by this process, are reinjected in the hæmolysed blood. The intravenous injection of 18 c.c. of blood so prepared into a cat of 1·9 kilos. weight had no other result than a slight permanent rise in the blood-pressure. The urine, which was, of course, stained with hæmoglobin, was somewhat increased in amount, probably owing to the rise in blood-pressure. It amounted to 1 c.c. in five minutes—a good value for an anæsthetised cat. In order to find out what degree of hæmolysis existed, the total hæmoglobin content in a sample of the blood was compared, by Sahli's method, with that free in the plasma. The latter was determined by diluting 1 c.c. of the blood with 99 c.c. of Ringer's solution, allowing the corpuscles to deposit and estimating the hæmoglobin in the supernatant liquid. It was found that 22 per cent. of the total hæmoglobin was free in the plasma. If all the corpuscles injected had been hæmolysed the calculated amount would be 20 per cent., so that it is evident that once freezing and thawing is effective for complete hæmolysis.

In another experiment the stromata were separated from the hæmolysed blood by the centrifuge, the deposit extracted with dilute alkali, neutralised and filtered. There was no fall of blood-pressure nor other toxic action when injected. Finally, the deposit on the filter-paper was suspended in Ringer's solution and injected, with no result.

It should be mentioned that in one case only out of many the injection of hæmolysed blood was found to produce intravascular clotting. This was clearly due to a peculiar individual sensibility to fibrin ferment.

Some interesting facts came out in another set of experiments in which hæmolysis was produced *in vivo* by the intravenous injection of warmed tap-water. A volume of water equal to the calculated volume of the blood-plasma was introduced slowly. In one case, in which seventy-two minutes were taken for the injection, a sample of blood taken five minutes after the end of the injection showed a total hæmoglobin content of 98·8 per cent. of the original value, so that practically all of the water injected had left the circulation. There was no fall in the blood-pressure. Indeed, it seemed to me that water was more effective in maintaining the blood-pressure than a saline solution. Notwithstanding the fact that there was no fall in the blood-pressure, the rate of the urinary flow was only one-quarter of its initial rate. It contained much hæmoglobin, in concentration one-third of that in the plasma. The percentage of hæmolysed corpuscles was found to be between one-fifth and one-sixth of the whole. In another similar experiment there was no actual diminution in the renal secretion—rather a slight increase, but very much less than would have resulted from an equal volume of Ringer's solution. That this action on the kidney was in some way connected with the dilution of the salts of the plasma, and not with the presence of hæmoglobin in solution, is shown by the result of adding sodium chloride to the blood in circulation. Although the water injected had left the blood-vessels, it is to be remembered that the total salt concentration of the blood and tissues was distributed over a much larger volume of liquid than normally, so that the plasma would still be hypotonic. The amount of sodium chloride required to make the water introduced into a 0·9 per cent. solution was dissolved in a very small quantity of water and injected into a vein. Although this was done very slowly the result was a rather large fall of blood-pressure—a common result in my experience of hypertonic saline solutions. This fall was recovered from in the course of twenty minutes, and the urinary flow was then raised to three times its original rate. It may be noted that Regéczy (11) injected large quantities of water into the veins of dogs without apparent harm. The explanation of the decreased urinary secretion when the salt-content of the plasma is below normal may possibly be due to the increase of osmotic pressure of the plasma proteins, thus reducing the effective filtration pressure in the glomeruli. I found (12) that the osmotic pressure of the proteins of ox-serum was 36–40 mm. of mercury in the presence of Ringer's solution, 116 mm. in water.

It is well known that certain chemical substances, such as nitrites, pyrogallol and other benzene derivatives, bile-salts and so on, change a part of the hæmoglobin into methæmoglobin, shown by the brown colour of the blood. I tested the action of some of these, namely pyrogallol, aniline acetate and bile-salts. They showed themselves to be toxic by the fall of blood-pressure and certain nervous effects. In the case of pyrogallol, which was given in the somewhat excessive dose of 0·8 grm. per kilo., the respiratory centre was paralysed. The blood became brown in colour but there was no actual breaking up of the corpuscles. The urine was greatly reduced in amount but normal in colour. The reduction in the kidney secretion was evidently due to the lowered blood-pressure, because it was restored more or less by the injection of gum-saline, which had the effect of a partial restoration of the blood-pressure. There appears to have been sufficient unaltered hæmoglobin to convey oxygen to the tissues if it was carried round fast enough by increasing the volume of the blood. Since there was no actual hæmolysis in these experiments, they only bear indirectly on the question at issue. Saponin, which does actually cause the passage of hæmoglobin into the plasma, was found to stop the heart before any notable degree of hæmolysis had been produced.

Although methæmoglobin can be reconverted into hæmoglobin by a chemical reducing agent, it appears that merely reducing the oxygen tension is incapable of doing this. When once formed it is a useless substance in the body and has to be got rid of.

Hæmolysis in itself appears, then, to be harmless. The ill-effects ascribed to it must be due to other concurrent phenomena. For example, the results of transfusion of incompatible blood are probably due to a reaction to foreign protein, analogous to that of anaphylactic shock. In this connection the statements of Schmidt and Bennett (13) are of interest. They show that hæmoglobin itself is unable to act as an antigen, whereas the "globin" obtained from it by chemical agency has this property. Brodie (14) found that foreign blood is less effective in the perfusion of isolated organs, especially the heart, than that of the same animal is. There is produced in some way an obstruction to the flow. Cushny and Gunn (15) showed that serum and egg-white have a peculiar constricting effect on the coronary arteries of a heart that has been previously perfused with a pure saline solution. Yanagawa (16) showed that the property is associated with the albumen of the serum, not with the globulin. Of course hæmoglobin in solution in the plasma is lost by the urine, and if there is a fall in blood-pressure from other causes, as by loss of fluid from the blood, there may be obstruction of renal tubules. The practical conclusion to be drawn is, obviously, that the blood-pressure must be kept up by intravenous injection, not by vaso-constrictor drugs, which really reduce the supply to the capillaries.

Although hæmoglobin shows itself to be innocuous when free in the plasma, it does not necessarily follow that it can serve its normal purpose of carrying oxygen. It is true that Barcroft and his coadjutors have been unable to find any difference in the relations of hæmoglobin to oxygen, whether they investigated the corpuscles or a solution.

It is well known that a solution devoid of hæmoglobin is insufficient to supply enough oxygen to many organs, especially glands, when used for artificial perfusion. Dale and Richards (9) found also that the normal dilator reaction of the capillaries to histamine was absent unless red blood-corpuscles were present in the perfusion fluid. I have used this latter fact as a test for the oxygen-carrying capacity of dissolved hæmoglobin. Cats were used for the experiments. Blood was first removed from a cannula in one of the carotid arteries to the extent of about half the calculated volume in the body. It was immediately replaced by gum-saline in order to keep the animal alive. The blood withdrawn was defibrinated by whipping and hæmolysed by the addition of twice its volume of a 6·5 per cent. solution of gum acacia in distilled water. Sodium chloride was then added in sufficient amount to make the gum solution present up to 0·9 per cent. The mixture was strained through glass wool and adrenaline added to make one part in five millions, as shown by Dale and Richards to be necessary to give tone to the capillaries. The femoral artery and vein, having been already prepared, were tied, a cannula, previously connected with the perfusion apparatus, and containing the oxygenated hæmoglobin solution, rapidly inserted, and the vein opened to allow the fluid to escape. The cat was then killed by opening the heart, and the leg inserted into a plethysmograph containing warm water, and arranged to trace on a smoked surface by means of a float recorder in the usual manner. The injection of 0·1 mgrm. of histamine into the rubber tube near the cannula was followed in some cases by an obvious dilatation of the limb, showing that dissolved hæmoglobin acts as an efficient oxygen carrier. Whether it is equally efficient in this respect to the red corpuscles is not, of course, shown. It is to be remembered that, as Dale and Richards pointed out, the action of histamine shows itself in the plethysmograph as a difference between constriction of arterioles and dilatation of capillaries, and may therefore vary much in different cases. It seems, therefore, that unless there is actual decrease in volume we must suppose that the capillaries reacted. If the perfusion fluid contains no hæmoglobin the arterial constriction alone is seen. This was the case in one of my experiments, in which the circulation in the limb had been interrupted too long, owing to the death of the animal from an overdose of ether.

It was found that the excitability of the muscles on stimulation of the sciatic nerve was prolonged for a greater time when perfused with a solution containing hæmolysed blood, as compared with that of the opposite side. Although this observation may perhaps indicate no more than that perfused muscles retain their excitability longer than those through which no circulation takes place, it shows that hæmolysed blood was not toxic. The isolated mammalian heart, perfused with a solution containing salts and gum arabic, showed an increase in magnitude of beat when hæmolysed blood was added. But this was only temporary and in all these perfusion experiments the vasoconstrictor action of the serum-albumin, to which reference has already been made, sooner or later causes a great decrease in the rate of flow through organs when solutions containing whole blood, serum or plasma are used. In future experiments, the effect of blood-corpuscles which have been washed with saline before hæmolysis must be tested.

The advantage of the possibility of using hæmoglobin in solution in perfusion fluids is that, when corpuscles are added, there is a tendency for these to deposit in parts of the apparatus where its rate of flow is small or at a lower level. This is especially the case when gum acacia is present, as noted by Dale and Richards. The fact is of importance in two ways: the circulating fluid becomes more or less deprived of the advantage of the hæmoglobin, and, owing to the changes in viscosity, brought about by the irregular distribution of the corpuscles, observations on the rate of flow through perfused organs are made inaccurate.

SUMMARY.

Experiments with defibrinated or hæmolysed blood on the rabbit are not to be taken as applying to other animals, owing to the peculiar aptitude of this animal to intra-vascular clotting.

With extremely rare exceptions, in which intravascular clotting may occur, defibrinated and even hæmolysed blood from the same species are innocuous to the cat and dog.

If the blood-pressure is caused to fall by any means there may be reduction of renal secretion, but this is restored by the intravenous injection of gum-saline.

Hæmoglobin dissolved in the plasma is able to serve as a carrier of oxygen, but the experiments do not enable a statement to be made as to whether it is as efficient in this respect as the red corpuscles are.

It appears that the serious results of the transfusion of incompatible blood are not to be ascribed to the hæmolysis as such, but are rather an aspect of the action of foreign serum protein analogous to that responsible for anaphylactic shock.

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THE DETERMINATION OF THE REACTION OF BACTERIOLOGICAL CULTURE MEDIA.

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CONTENTS.

	PAGE.
Introduction	9
Estimation of the Hydrogen-ion Concentration	14
Sources of Error in the Colorimetric Method of Estimating the Hydrogen-ion Concentration of Media	15
Relation of the "Eyre Notation" to the Hydrogen-ion Concentration	22
Procedure recommended for the Preparation and Standardisation of Ordinary Broth	26
Discussion	27
Conclusions	29
References	30

INTRODUCTION.

The importance of a correct adjustment of the reaction of bacteriological media cannot be over-estimated if one wishes to obtain uniform growths or constant toxin production. The truth of this has been very clearly demonstrated in recent work on anaërobic bacteria.

In the ordinary course of bacteriological work one comes across from time to time a batch of media which, for some reason or another, fails to give satisfactory growths. Yet the individual constituents of the defective medium may be of the same quality as the good medium. In such cases it is not uncommon to find that the "reaction" of the medium is at fault, usually on the acid side. It is generally accepted that the reaction of the medium—when dealing with pathogenic bacteria—should correspond to that of the tissues. Atkin (1), using what he termed the neutral point of phenolphthalein (first perceptible darkening without any change of colour), showed that a common optimum of growth is obtained with broth of this reaction. In actual practice the production of a medium of a particular reaction is attended with considerable difficulty, and a good deal of work has been carried out to improve the technique of standardisation. Formerly the only test applied was litmus paper: a blue coloration was considered to indicate a satisfactory reaction. The crudeness of this method soon became apparent when one was dealing with the more delicate organisms, and accordingly measures were taken to remedy the defect. Schultz (2) appears to have been the first to advocate actual titration. His work was supplemented and improved by Fuller (3) in America, and Eyre (4, 5) in this country. The principle employed was to estimate by titration with N/10 soda the degree of acidity, using phenolphthalein as an indicator. The end-point of phenolphthalein is somewhat too alkaline for use, and therefore N/1 HCl was added to the "neutral" broth and the reaction was described as being, say, 10 c.c. N/1 HCl per litre, or shortly "+ 10."

Since the above methods were devised, however, great advances have been made in our knowledge of the "reaction" of fluids. Acidity means excess of H- over OH-ions, and alkalinity the reverse. Neutrality is merely the presence of equal numbers of H- and OH-ions per c.c. The purest re-distilled water is taken as the standard of absolute neutrality. According to Sörensen's scale, which is explained later in this paper, its value is P_H 7·07. Normal blood lies slightly on the alkaline side of this neutral point.

It is important in all investigations which deal with the standardisation of biological methods that a simple but sound system of nomenclature and symbols should be used. The exponential method, first suggested by Sörensen, is usually admitted to satisfy most requirements. The requisite amount of mathematical knowledge required is slight. It is hoped that the following remarks with reference to the ionic theory and the law of mass action will make clear the notation adopted.

The ionic theory was first propounded by Michael Faraday in 1833, and since then greatly extended by the work of Clausius (1857), Arrhenius (1887), Kohlrausch and many others. The theory was intended to furnish an intelligent explanation why some solutions were conductors of electricity and others not. Pure water scarcely conducts electricity at all, a solution of $CuSO_4$ extremely well, while it is believed that if water could be obtained absolutely pure it would be a non-conductor of an electric current. Faraday suggested that the electric current is carried by the dissolved matter in the solution. The current is led into the solution by a plate called the "anode," and conducted out by another plate, the "kathode." Now it is known that the anode acquires a positive electrical charge and the kathode a negative one.

Faraday suggested the dissolved salt $CuSO_4$ was split into two component parts, oppositely and equally charged with electricity, the Cu positively and the SO_4 negatively. This splitting up into two parts he called "ionisation." Evidently now the positive ions will be attracted to the kathode and the negative ions to the anode. The charge on each ion is neutralised by the electrodes, and so in each case the latter have to draw upon the source of electrical power for more electricity. This, therefore, constitutes the electric current. It has further to be supposed that the constituent ions have temporarily lost all chemical individuality, and that when they have had their charge neutralised they regain their chemical characteristics, and can be recognised as such, *e.g.* Cu by the familiar coppery deposit on the kathode, while the SO_4 attacks the water around the anode, unites with its hydrogen, regenerates H_2SO_4 and leaves behind un-ionised oxygen, which is found collected as bubbles on the anode. As water is slightly conducting one must suppose it is slightly ionised and so contains H- and OH-ions. Briefly, then, all conducting solutions are ionised. The ions are equally and oppositely charged, and in this condition their chemical properties are hidden.

The "reaction" of a medium.—From what has been said it will be seen that in an acid medium containing, say, HCl there must be H-ions and Cl-ions. Further, all acid media contain H-ions, and this constitutes the distinctive feature of any "acid" medium. Similarly any alkaline medium such as caustic soda NaOH ionises into Na- and OH-ions. In all alkaline media there exists an excess of OH-ions—again a distinctive feature. If both OH- and H-ions exist together in a solution, then the "reaction" would be determined by the nature of the majority, *e.g.* if H-ions are in excess then it is "acid," while if the OH-ions are in the majority it is "alkaline." Thus a "neutral" medium would be one in which there is an equal number of H- and OH-ions per c.c. It should be pointed out that the H-ions may come from several sources—inorganic acid, organic acid, or acid salt (*e.g.* $KHSO_4$).

Different acids, however, vary greatly in their relative strengths, depending upon their degree of ionisation. Thus HCl is highly ionised and thus is a "strong" acid, while acetic acid is "weak," because it is relatively slightly ionised. In like manner the degree of alkalinity depends on the extent of ionisation into hydroxyl- (OH-) ions. The comparative strengths of different acids is best expressed by means of their dissociation constants. In order to explain this we must refer to the Law of Mass Action.

Mass action.—Suppose two molecules A and B react to produce two other molecules C and D, then in many instances it is known that the action does not proceed indefinitely. When a certain percentage of A and B has been converted into C and D the reaction ceases. The amount changed depends on a variety of circumstances. Given a definite set of conditions then the percentage of A converted into C and D is definite and constant. This was expressed by Guldberg and Waage in 1867 in the form of a "law."

"The velocity of a reaction is proportional to the concentration of the reacting substances," *e. g.* if A is acetic acid and B is ethyl alcohol, then C is ethyl acetate and D is water. The reaction is written thus:

$$CH_3 \,.\, COOH + C_2H_5 \,.\, OH \rightleftarrows CH_3 \,.\, COOC_2H_5 + H_2O.$$

In this case two-thirds of the acid are converted and no more. As a simpler illustration we may quote $H_2O \rightleftarrows H + OH$. This law is capable of mathematical expression, and we direct attention to this in order to show the meaning of the symbol "P_H," or, as it is sometimes written, "pH." Let the concentrations (*i. e.* mass per unit volume) of A, B, C, D be represented by (A), (B), (C), (D). Also let V_{AB} represent the "velocity" of the reaction between A and B to produce C and D, *i. e.* mass converted per unit time. Similarly V_{CD} denotes the rate of conversion of C and D into A and B.

Therefore—

$$V_{AB} \propto (A)\ (B),\ i.\,e.\ V_{AB} = \lambda\ (A)\ (B),$$
$$V_{CD} \propto (C)\ (D),\ i.\,e.\ V_{CD} = \mu\ (C)\ (D),$$

λ and μ being constants.

When the reaction is at an end, the velocity of change of A and B into C and D must, by the kinetic theory, equal that of C and D into A and B.

So ultimately $V_{AB} = V_{CD}$, *i. e.* $\lambda\ (A)\ (B) = \mu\ (C)\ (D)$.

Therefore $(A)\ (B)\ (C)\ (D) =$ a constant.

Applying this to the case which concerns us most, viz. $H_2O \rightleftarrows H + OH$ we have—

$$\lambda\ (H)\ (OH) = \mu\ (H_2O) \text{ or } (H)\ (OH) = k\ (H_2O),$$

where k is a constant.

Now the concentration of H_2O molecules is great and is relatively unaltered. It is usual to regard it as constant, and so we may write $(H)\ (OH) = k\ (H_2O) = K$, which is called the "dissociation constant."

The value of K has been found by various means to be approximately $10^{-14 \cdot 14}$ at 18° C. Now, in a perfectly neutral solution (*i. e.* absolutely pure water) there must obviously be as many H- as OH-ions. It therefore follows that $(H) = (OH)$, and so $(H) \,.\, (OH) = (H)^2$, *i. e.* $(H)^2 = 10^{-14 \cdot 14}$, and $(H) =$ the square root of $10^{-14 \cdot 14} = 10^{-7 \cdot 07}$. In an acid solution the (H) must be greater than $10^{-7 \cdot 07}$, of course in an algebraic sense, while the (OH) is less than $10^{-7 \cdot 07}$, the product of the two always being $10^{-14 \cdot 14}$. It will also be seen that $(H) = K/(OH)$ or $(H) = 10^{-14 \cdot 14}/(OH)$ and $(OH) = 10^{-14 \cdot 14}/(H)$.

For purposes of measurement or the tabulation of results it is only necessary to deal with hydrogen-ion concentrations. The OH values can always be deduced from them if required.

The values quoted above for hydrogen-ion concentrations are always negative powers of 10. In all calculations one would naturally use ordinary logarithms. Sörensen (6) suggested that these indices (with the negative sign dropped) should be used to designate the ionic concentrations of the solutions being dealt with. This saves a great deal of space and is exceedingly convenient. To the non-mathematical reader, however, it presents some difficulty.

The symbol chosen for this index of the hydrogen-ion concentration (with negative sign understood) is P_H. A few examples may make the idea clear:—

The H-ion concentration of pure water has been shown to be $10^{-7·07}$. Thus its P_H is 7·07.

Again, N/100 HCl solution should contain ·01 grm. of hydrogen in a litre. This is only true if all the hydrogen were present as ions. Ostwald, however, showed that ionisation varied with the degree of dilution. It has been found by various means that at room temperature only 96 per cent. of the hydrogen is ionised. So the H-ion concentration of N/100 HCl is 96 per cent. of 10^{-2}, *i. e.* ·0096. Now the "logarithm" of ·0096 is $-3 + 0·98$, which is written $\bar{3}·98$, but it is easier to use the negative index $^{-2·02}$. So the H-ion concentration of N/100 HCl is $10^{-2·02}$ or $P_H = 2·02$ if the negative sign is understood.

Further, N/10 NaOH has a H-ion concentration of $10^{-14·14}/10^{-1}$, *i. e.* $10^{-13·14}$. But only 84 per cent. is ionised. So this alters the value to $10^{-14·14}/\frac{84}{100}$ of 10^{-1}, which is very approximately $10^{-13·06}$. Hence P_H of N/10 NaOH is 13·06.

Buffer solutions.—It is obvious that the addition of even a minute quantity of well-ionised acid or base must easily alter the P_H value of water. But if one uses other solutions instead of water, then the above generality does not always hold. For example, a little HCl added to a sodium acetate solution produces surprisingly little alteration in the P_H value. On investigation it is found that the cause is the fact that the acetic acid produced ionises with difficulty. That is, the H-ions are used up by the CH_3 COO and so the mixture does not show much increase in P_H. Such solutions are called "buffer solutions." Other examples are phosphates, citrates, borates, proteins and amino-acids.

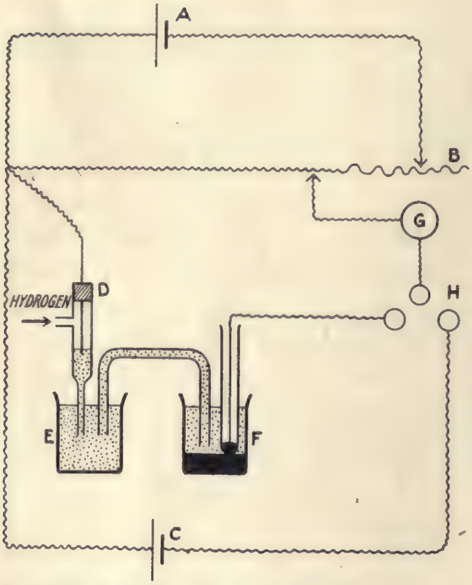
Chemical titration.—In ordinary titration by chemical means one assumes that x c.c. of N/10 acid are neutralised by x c.c. of N/10 alkali. This is so as regards total effect, but it is no measure of the "acidity" or "alkalinity" of the solutions used. In the end, the total amount of acid and alkali is neutralised, but it gives no idea of the scarcity or abundance of ions present. During the process HCl is freely ionised and so its neutralisation is accomplished "*en bloc*" as one might say. On the other hand, if the acid were CH_3 COOH the ionisation is very poor. In this case neutralisation proceeds in instalments. Or it might be expressed thus: as fast as the ions are neutralised, fresh undissociated acetic acid molecules proceed to break up, giving more H-ions, in an effort to keep the H-ion concentration of the solution constant. This goes on till all the acid present has been completely dissociated. It is this concentration of ions which is so important in considering the reaction of bacteriological media, and therefore it is only by going back to a truly fundamental method of measurement that one is enabled to estimate the character of a medium.

Therefore we propose the correlation of Eyre's scale of acidity, obtained by chemical titration, with a P_H scale. This is developed in greater detail further on in the paper.

ESTIMATION OF THE HYDROGEN-ION CONCENTRATION.

In the estimation of the hydrogen-ion concentration of different fluids two methods are commonly used — (*a*) the hydrogen electrode, (*b*) the colorimetric.

(*a*) *The hydrogen electrode method.* —This depends on the fact that there exists a definite potential between any metal and its salts. Here we are dealing particularly with hydrogen (electrolytically a metal) and its acids (really salts of hydrogen). If, therefore, we have an electrode coated with platinum or palladium to occlude the hydrogen, and immersed in an acid or alkaline solution, it follows that a definite E.M.F. exists between this hydrogen electrode and its solution. The E.M.F. varies with the strength of the solution, *i.e.* with the H-ion concentration. To any value of the E.M.F. there corresponds a definite value of the H-ion concentration. Full details of this method are to be found in Sörensen's (6) and Michaelis' (7) articles.



A. ACCUMULATOR FOR MAIN CIRCUIT
B. ADJUSTABLE RESISTANCE
C. STANDARD WESTON CELL
D. HYDROGEN ELECTRODE
E. SATURATED KCl SOLUTION
F. CALOMEL ½ ELECTRODE
G. CAPILLARY ELECTROMETER
H. SWITCH

FIG. I.

This method is carried out by the use of a potentiometer and hydrogen electrode. A short description of the apparatus and technique is given:

(1) The electrode was of the "upright" type (Walpole (8)), of capacity about 1 to 2 c.c., which could be adjusted as desired.

(2) The potentiometer was kindly lent by Dr. H. H. Dale, F.R.S., of the *Medical Research Committee*, to whom we offer our best thanks for its use.

(3) The standard half-cell was of the saturated calomel type. It was made by us with great care of the purest materials.

(4) The connecting solution was saturated KCl. It was chosen because it gives the least difference of contact potential, and is the easiest to set up and maintain.

(5) The point of balance was determined by aid of a capillary electrometer.

(6) Hydrogen was generated by electrolysis of water.

The general arrangement of the apparatus was as shown in Fig. 1.

A standard cell (Weston), as shown in the figure, is also inserted into the apparatus so that the readings of the hydrogen electrode may be at once standardised. The instrument reads E.M.F. to ·001 volts. This E.M.F. is translated into H-ion concentration or P_H values by means of a table, graph, or formula.

(*b*) *Colorimetric method of estimating* P_H.—Indicators change their colours with change in P_H values. The limits of change for any one indicator are sometimes fairly sharp, while for another they may be relatively broad. As, however, there are many scores of indicators each with its own so-called "neutral" point it will be seen that it is quite possible to cover a wide range in P_H values by a suitable choice of indicators. Various devices have been used to secure forms of apparatus for determining with tolerable accuracy the P_H value of any given solution. Obviously the results are not so good as the use of the hydrogen electrode apparatus, but at any rate they are rapid, and easy to work with.

SOURCES OF ERROR IN ESTIMATING THE H-ION CONCENTRATION OF MEDIA.

We here give the routine scheme formerly used in this Laboratory for the preparation of media. In each case the basis is a watery extract of meat. From this the ordinary broth is prepared and from it the solid media.

(1) Ordinary broth: (*a*) 1 lb. of meat free from fat is minced, and then macerated in cold water (1 litre) overnight. As an alternative to this the mixture may be macerated at 40° C. for half an hour.

(*b*) Boil for ¼ hour over a gas-ring, filter through muslin and then through paper. Make up 1000 c.c. and add 10 grm. peptone (Morson or Witte) and 5 grm. salt. Dissolve and steam for 20 minutes. The medium should now be ready for standardisation.

(*c*) Measure 10 c.c. broth into a porcelain evaporating dish. Add 25 c.c. distilled water. Have a control dish alongside prepared in exactly the same manner. Add 5 drops of ½ per cent. alcoholic solution of phenolphthalein to the first dish. Warm it over gauze by a Bunsen burner. When boiling run in N/10 NaOH from a burette. Stir continuously and look for a slight but definite darkening of the liquid. This might better be explained as the first but distinct change in tint (faint rose, or, as some call it, a salmon tint) of the fluid as compared with that of the control. No distinct pink should appear. A good test for the end-point is obtained by allowing the liquid to cool, when a definite pink shade should appear. Further, just before the end-point is reached a precipitate of phosphates occurs. Note the quantity of N/10 NaOH required to produce this. With these data a calculation is made as to the amount of N/1 soda required to produce the same reaction in the rest of the broth. This amount of soda is then added and the flask shaken.

(*d*) The neutral broth is now steamed for half-an-hour to deposit the phosphates and then filtered free from them. The reaction has now to be adjusted by the addition of 5 c.c. of N/1 HCl per litre of broth. With the above "end"-point we found that media corrected to + 5 had reactions corresponding to that of plasma P_H 7·6.

(*e*) Lastly, it is placed in tubes or flasks and sterilised by heat in the autoclave for twenty minutes at 115° C.

(2) Glucose broth is made from the above by the addition of 1 per cent. of glucose.

(3) Ordinary agar: To the ordinary broth add 2 per cent. of powdered agar. In making the agar medium the agar powder should be emulsified in a mortar with the broth and then steamed to dissolve it completely.

The above details may be followed with minute care and yet the final product is sometimes unsatisfactory as regards its reaction.

This may be due to hydrolytic changes which occur during sterilisation and lead to an increase of acidity. Or again, the process of titration may be in error quite apart from accidental errors (arithmetical calculation). The most important in this respect, as we shall show later, arises from the difficulty in judging accurately the "end-point" of the indicator selected.

Further, there is a possibility of an error in P_H arising in bringing the medium back to the reaction selected, and there is a question whether media should be titrated at the boiling-point or at room temperature.

We have attempted to analyse each of these points, and in addition we have endeavoured to devise a method whereby such errors may be eliminated or avoided, thereby yielding media which are quickly and yet accurately standardised.

Alteration of the Reaction due to Heating.

All workers on this subject have stated that under the influence of heat bacteriological media tend to become more acid in reaction. But the extent to which this occurs with different media when sterilised in the ordinary manner (by steaming or autoclaving) is not very definite. For instance, Eyre states that after three steamings for three-quarters of an hour the reaction becomes constant in the case of meat extract. Anthony and Ekroth (9), on the other hand, found that "the state of complete hydrolysis, *i. e.* the point at which no further acidity is produced, was not reached with meat infusions (which had been previously subjected to boiling over the open flame for one to two hours) even after prolonged autoclaving at 15 lb. pressure for eight hours." Clark (10) found that a meat infusion containing ½ per cent. K_2HPO_4 brought to a reaction of P_H 7·19 before sterilisation, changed to P_H 6·70 after sterilisation. The change in reaction was accompanied by a heavy precipitate.

In the experiments quoted below we have confined our observations to the effects of what might be regarded as the maximum amount of heat employed, under ordinary conditions, to sterilise the media. In the case of sterilisation by steaming, the effects have been restricted to three steamings of 20 minutes each. The same batch of media was also autoclaved at 115° C. for 20 minutes. In some instances the media were autoclaved on two more occasions, since in preparing media it is at times necessary to re-autoclave.

Comparison of the Effects of Three Successive Steamings of Ordinary Broth (for half an hour each), with Three Autoclavings of 20 Minutes each.

Steaming.

Reaction before.		Reactions after.					
		1st		2nd		3rd	
E.M.F.	P_H.	E.M.F.	P_H.	E.M.F.	P_H.	E.M.F.	P_H.
0·713	8·01	0·688	7·57	—	—	—	—
?		0·690	7·61	0·685	7·52	0·681	7·45
0·733	8·35	0·723	8·18	0·711	7·98	0·706	7·88
0·692	7·64	0·691	7·62	0·690	7·61	0·688	7·57
0·722	8·16	0·731	8·32	0·729	8·28	0·722	8·16
0·734	8·37	0·732	8·34	0·730	8·30	0·728	8·26
0·712	8·12	0·718	8·10	0·717	8·08	0·716	8·06
0·729	8·28	0·726	8·23	0·722	8·16	0·719	8·12

Autoclaving.

Reaction before.		Reactions after.					
		1st		2nd		3rd	
E.M.F.	P_H.	E.M.F.	P_H.	E.M.F.	P_H.	E.M.F.	P_H.
0·733	8·36	0·726	8·23	0·714	8·02	0·710	7·96
0·692	7·64	0·687	7·55	0·685	7·52	0·685	7·52
0·722	8·16	0·733	8·35	0·722	8·16	0·720	8·12
0·734	8·37	0·728	8·26	0·714	8·12	0·713	8·01
0·717	8·12	0·714	8·02	0·708	7·92	0·704	7·85
0·729	8·28	0·714	8·02	0·693	7·66	0·667	7·20

After three steamings the average variation in H-ion concentration is about

P_H 0·13, while after the same number of autoclavings the figure is 0·23. The total variation in reaction due to sterilisation in the case of ordinary broth is therefore very small, and not likely to interfere with bacterial growth, provided, of course, one starts with a reaction which is on the alkaline side of neutrality.

Repetition of the Experiment with 1 *per cent. Glucose Broth.*

Steaming.	E.M.F.	P_H.
Reaction before . .	·729	8·28
Reaction after (1st) .	·726	8·23
,, (2nd) .	·722	8·16
,, (3rd) .	·719	8·11

Autoclaving.	E.M.F.	P_H.
Reaction before . .	·729	8·28
Reaction after (1st) .	·713	8·00
,, (2nd) .	·693	7·66
,, (3rd) .	·666	7·19

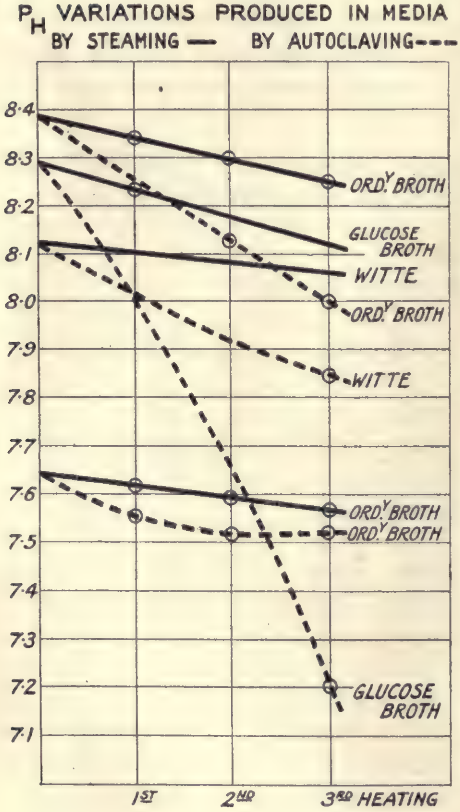


FIG. 2.

With carbohydrate-containing media, the results indicate clearly that there is a considerable tendency to the production of acid during the process of sterilisation by heat. This, of course, is no more than one would expect from the fact that hydrolysis of carbohydrate into smaller molecules proceeds more rapidly with rise of temperature or prolongation of action. In the case of glucose broth this is very well marked after autoclaving; great disruption of the molecule must occur. In fact, after an autoclaving the reaction can change by as much as P_H 1·12. Obviously, under these circumstances, sugar-containing media should never be autoclaved. Agar itself is neutral in reaction, so that the addition of it to ordinary broth will tend to lower the alkalinity of the finished product, and agar made with broth of a reaction of P_H 7·8 will probably have a reaction of P_H 7·4. Thus the final reaction of the agar should be carefully checked.

The above experimental values are plotted to show visually the difference in the effects of repeated steamings *versus* autoclavings (see Fig. 2). Ordinary broth and agar vary but little, as will be seen by either steaming or autoclaving, but glucose is seriously decomposed, while three steamings are never so destructive as one autoclaving. The application therefore of any unnecessary heat in the preparation of bacteriological media, and especially when carbohydrates are present, is to be avoided. Failure to observe this will certainly result in upsetting the final reaction.

Difficulty of Judging the End-point of the Indicator selected.

Leaving out of account the possibility of accidental errors such as arise from measuring or calculation, we think that the most frequent source of error arises in adjudging correctly the end-point selected. To a certain extent this trouble exists with most indicators, but is more marked with some than with others. The selection of an arbitrary shade of colour, or the matching of two colours, as is the usual practice, allows of a great possibility for errors. Thus an indicator should be selected which not only changes at the reaction required, but which gives a constant and easily recognisable "end-point."

We have tried a series of indicators including rosolic acid, neutral red, litmus, cresol red, thymol blue, tetra-brom-phenolphthalein, phenolphthalein thymolphthalein, etc., in our attempts to find the best with which to titrate media.* In our hands phenol- and thymolphthalein proved the most suitable. With both of these the change is from a colourless to a coloured solution, while the change takes place within a slight range of hydrogen-ion concentration—in fact, within a half P_H with the first indicator and rather less with the second.

The objection has been raised that these two phthalein indicators change colour at a point on the alkaline side of neutrality. But in bacteriological work this is really no objection, as it is necessary during the process of preparation to bring the meat extract to an alkaline reaction in order to get rid of the excess of phosphates. If one does not do this the broth will not be clear and the phosphates will be constantly precipitating out, which, from the practical point of view, is a considerable objection.

We shall deal first with *phenolphthalein*, as it was with this indicator that the majority of the comparative tests were made. Phenolphthalein, as anyone familiar with bacteriological work knows, has been almost universally used in the titration of media; but there is a lack of uniformity as to the end-point, and a great variety of arbitrary shades of colour have been advised by different writers. The end-point we have used corresponds to the first development of a trace of pink or a salmon tint in the medium, and not to the development of a *distinct* pink colour. With the broth diluted about three times this change is fairly sharp, if one uses another dish alongside as a control, as already described. According to Clark and Lubs (11), the addition of water does not appreciably alter the H-ion concentration, and we also found that dilution did alter the reaction to a measurable extent. This end-point or "neutral point" can be judged much more exactly than any arbitrary shade of pink.

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* The indicator dyes which we have used were prepared and supplied to us by the Cooper Laboratory, Watford.

When titration is carried out at 100° C., the first distinct change of shade or development of a faint trace of pink with phenolphthalein corresponds to P_H 8·0–8·1 (tested after medium allowed to cool). The P_H reactions of the various pinks and reds used by bacteriologists are really very variable, as at that stage it requires a considerable amount of alkali to make any perceptible change in the shade of the colour. In fact this definite pink can correspond to any P_H from 8·2 to 8·7 or even 9·0.

The following results show the reaction of our "neutral point" of phenolphthalein (faintest pink or salmon) when titrated at 100° C.:

Observer.	E.M.F.	P_H.
J. G.	·712	8·00
J. M.	·723	8·18
,,	·715	8·05
,,	·719	8·11
J. S.	·725	8·20

This gives an average of P_H 8·10.

So far our experience shows that with *thymolphthalein* even more accurate estimations can be obtained. This is due to the ease with which its "neutral point" can be judged. The neutral point is taken as the first perceptible darkening of the medium on the addition of N/10 soda. The titration is carried out exactly in the manner described for phenolphthalein, *but at room temperature*. Ten c.c. of the broth to be used is placed in a porcelain evaporating dish and 25 c.c. of distilled water added. A control dish is prepared in exactly the same manner. Add 5 drops of a ½ per cent. alcoholic solution of thymolphthalein to the first dish. Then run in N/10 NaOH from a burette. Stir continuously and look for a slight but definite darkening of the liquid. This might more aptly be described as the disappearance of the yellow tint and the development of a bluish tinge. The change in the medium occurs quite sharply, and at times a single drop of soda in excess will change the medium to a greenish-blue colour. To titrate media the average of at least three readings should always be taken, and the first reading, in which a blue colour should be sought, rejected. Two factors are probably at work in the production of this sharp end-point, namely the relative sensitivity of thymolphthalein to a slight excess of alkali after a certain ionic concentration has been reached, and secondly to the change of colour, which is from a yellowish to a blue tint.

The relative ease with which this end-point can be reached is well shown in the following results obtained by different observers. In every case 10 c.c.

of broth was diluted with 25 c.c. of distilled water, and 5 drops of 0·5 per cent. alcoholic solution of thymolphthalein added.

Sample A.

N/10 soda required.	E.M.F.	P_H.	Observer.
3·6 c.c.	·775	9·08	J. G.
3·5 c.c.	·771	9·05	,,
3·4 c.c.	·770	8·99	J. M.

Sample B.

N/10 soda.	E.M.F.	P_H.	Observer.
4·2 c.c.	·776	9·09	J. G.
4·1 c.c.	·771	9·01	,,
4·1 c.c.	·768	8·96	J. M.
3·8 c.c.	·760	8·81	,,
3·9 c.c.	·762	8·85	M. H.
3·8 c.c.	·760	8·81	,,
4·1 c.c.	·778	9·13	L. G.
3·8 c.c.	·764	8·89	,,

The average P_H worked out at 8·95. The last two observers had never used this reagent before, yet their results correspond very closely with the other findings.

The objection, that such a reagent as thymolphthalein necessitates the addition of an excess of alkali, has already been dealt with, while a series of experiments showed that the addition of 10 c.c. of N/1 HCl per litre was sufficient to correct this excess and leave the final reaction of the medium, after sterilisation, at about P_H 7·6. (Fig. 4 is a graph by which the amount of acid necessary to bring the final reaction of the medium to any desired reaction can be found.)

Titration at the Boiling-point or at Room Temperature.

According to routine technique, titration with N/10 NaOH is performed at the boiling-point. The question arises as to whether this is justifiable and to what extent, if any, it interferes with the end-point. We have endeavoured to settle this matter by a determination of the P_H value at different temperatures. *A priori* one would easily imagine that at higher temperatures there should be greater dissociation and hence greater acidity. This was found to be the case but only when the liquid was *partially* dissociated in the cold. When substances like HCl or NaOH are tested there is no increase of acidity on heating.

Broth.	Sample	1	2	3	4	5
Hot (70° C.)	E.M.F.	·753	·748	·764	·753	·746
	P_H	8·69	8·61	8·89	8·69	8·58
Cold (room temperature)	E.M.F.	·690	·673	·730	·726	·704
	P_H	7·60	7·31	8·30	8·23	7·85
Difference	P_H	1·09	1·30	0·59	0·46	0·73

N/10 NaOH, N/10 HCl, and acetate mixture gave no appreciable difference whether hot or cold.

This explains why the broth as normally titred out becomes pink on cooling. There is less dissociation, or we may say that some of the dissociated molecules at the boiling-point have reunited to form un-ionised molecules. Obviously, then, one should titre at room temperature, or as near as possible the temperature at which the organisms are grown.

Using our neutral end-point we found that phenolphthalein did not give a sufficient degree of alkalinity when the titration was carried out in the cold. The reaction of the media in this case was rather under P_H 8·0. With thymolphthalein, however, a sufficient degree of alkalinity is easily obtained with this end-point as has already been indicated.

RELATION OF THE EYRE NOTATION TO THE HYDROGEN-ION CONCENTRATION.

After neutralisation it is necessary to bring media to a degree of alkalinity approximating to that of plasma (P_H 7·6). This is done by the addition of acid to the "neutral" broth. Eyre's method is to add 10 c.c. of N/1 HCl to every litre of broth which has been brought to his "end-point" of phenolphthalein. With our "end-point" the necessary amount of acid in the case of phenolphthalein is 5 c.c. and with thymolphthalein 10 c.c.

The addition of this acid, however, can only give a constant P_H to media of constant composition. It does not necessarily follow that if two samples of medium have a different composition the addition of an equal quantity of acid will ensure the same P_H.

The following experiments will show that there is a constant relation between Eyre's scale and P_H when the medium is constant.

Experiment I.—In the first experiment increasing amounts of N/1 HCl corresponding to 1 to 10 c.c. per litre were added to ordinary peptone broth. The reaction of the broth was neutral to phenolphthalein at 100° C. (Fig. 3).

Results as follows were obtained:

Amount of N/1 Acid added per Litre.

	0	2 c.c.	4 c.c.	5 c.c.	6 c.c.	8 c.c.	10 c.c.
E.M.F.	·737	·725	·714	·708	·704	·692	·682
P_H	8·42	8·22	8·02	7·92	7·85	7·64	7·46

P_H VARIATIONS IN BROTH ON ADDITION OF ACID

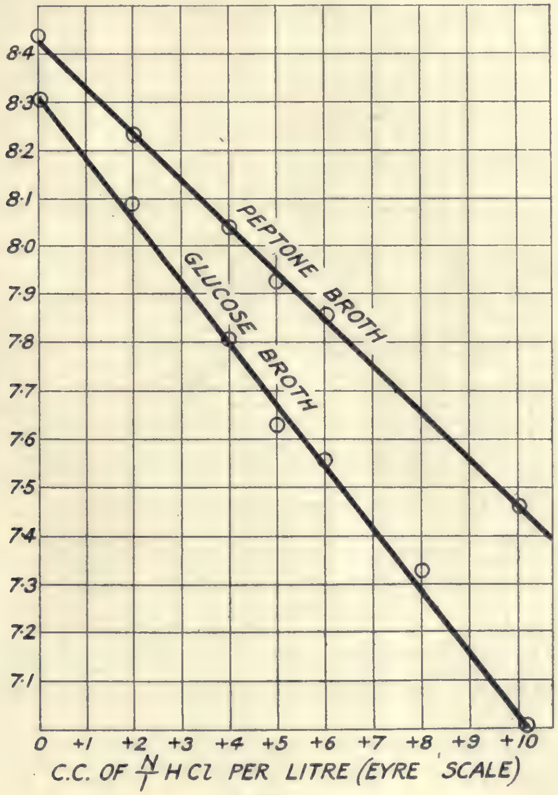

FIG. 3.

With 2 per cent. glucose broth very slightly different results were obtained :

Amount of N/1 Acid added per Litre.

	0 c.c.	2 c.c.	4 c.c.	5 c.c.	6 c.c.	8 c.c.	10 c.c.
E.M.F.	·729	·717	·701	·691	·687	·673	·654
P_H	8·28	8·08	7·80	7·62	7·55	7·32	6·98

Experiment II.—In this experiment broth which had been made neutral to thymolphthalein at 15° C. was used (Fig. 4).

Amount of N/1 Acid added per Litre.

	0 c.c.	2½ c.c.	5 c.c.	10 c.c.	15 c.c.	20 c.c.
E.M.F.	·765	·749	·739	·702	·670	·637
P_H	8·90	8·66	8·45	7·82	7·25	6·68

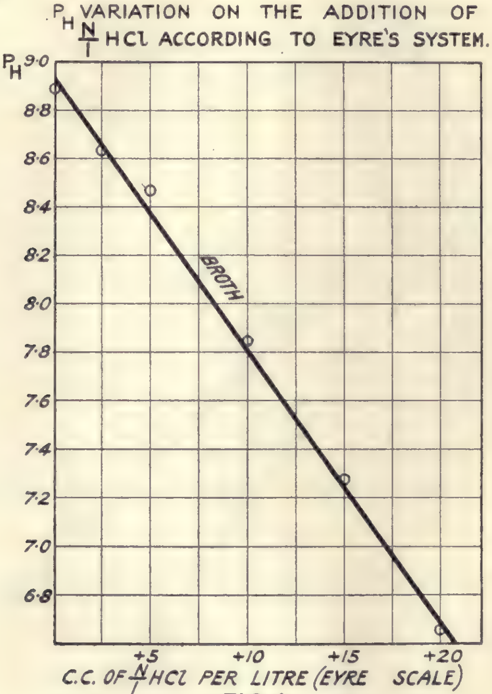

FIG. 4.

Experiment III.—In this experiment a parallel series was tested, the first made neutral to thymolphthalein and the second neutral to phenolphthalein.

Amount of N/I Acid added per Litre of Broth Neutral to Thymolphthalein.

	0 c.c.	5 c.c.	10 c.c.	15 c.c.	20 c.c.	25 c.c.	30 c.c.
E.M.F.	·755	·731	·703	·675	·652	·623	·602
P_H	8·73	8·32	7·83	7·34	6·94	6·43	6·06

Broth Neutral to Phenolphthalein.

E.M.F.	·740	·712	·690	·662	·635	·607	·580
P_H	8·48	8·00	7·60	7·12	6·65	6·15	5·70

Averaging the results obtained with ordinary broth, prepared in the manner described in the first part of the paper, it would appear that there was a constant relation between the P_H and the amount of acid added. In fact the addition of 1 c.c. of N/1 acid per litre makes a change of 0·1 P_H in the reaction.

Working on this basis it is possible to construct a graph by which one can find the H-ion concentration of broth from the amount of acid added, provided, of course, one knows the exact H-ion concentration of the broth previous to the addition of the acid, and that the medium has been made according to formula. Fig 5 has been so constructed by averaging the results of several readings.

If one uses a broth made according to another formula the relation between the acid added and the end-reaction is upset, as experiment No. 4 shows.

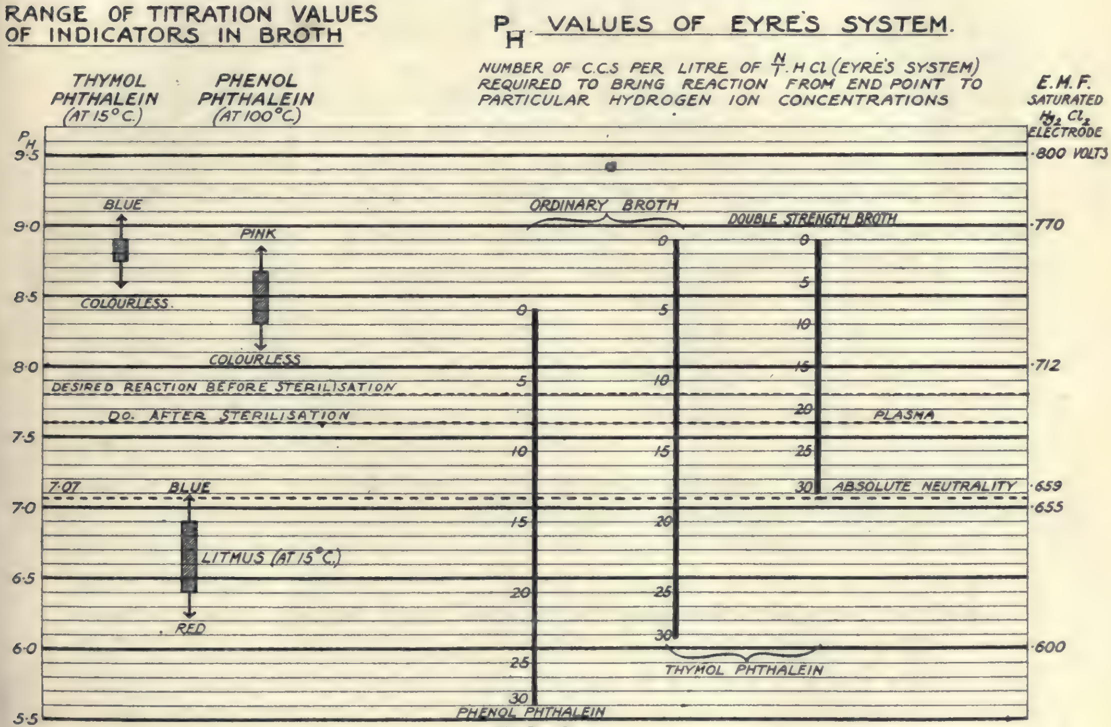

FIG. 5.

Experiment IV.—The broth on which the observations were made, was made up in double strength—that is, in making the meat extract, twice the quantity of meat was used and 2 per cent. of peptone added. The preparation of double-strength broth is frequently employed in making up our media. It is a simple way of making up nutrient agar, as the agar is first dissolved in water and then added to an equal volume of the double-strength broth.

Amount of N/1 Acid added per Litre.

	0 c.c.	5 c.c.	10 c.c.	15 c.c.	20 c.c.	25 c.c.	30 c.c.
E.M.F.	·766	·757	·742	·727	·703	·688	·665
P_H	8·92	8·76	8·51	8·25	7·83	7·57	7·18

Thus instead of 5 c.c. of N/1 acid per litre making a difference of 0·5 P_H, it makes a difference of 0·3 P_H.

These results are of great practical value, as it means that the older chemical titration of media in conjunction with a graph can give an accurate indication of the H-ion concentration, and so overcome one of the chief objections to the use of the purely chemical method. It is, however, necessary that the media be prepared in a constant manner and the initial reaction known. We have found that the P_H values obtained by graphs from the Eyre system are sufficiently reliable for the standardisation of media, especially when controlled in bulk.

To check the reaction in bulk it is not necessary to titrate out the broth again, but only to show that it lies between two points. This can be done most conveniently by a suitable choice of indicators. Theoretically, the indicator points should lie as near as possible to P_H 7·60, but the lack of suitable indicators prevents this. Comparison against a known colour shade might be used, but the technical difficulties in preparing the necessary solutions and their non-permanence make them almost impracticable for ordinary routine. As a quick and reliable procedure one cannot do better than use two indicators with reaction points sufficiently near P_H 7·60. After a series of tests we have found that cresol-red and phenolphthalein give satisfactory results.

In order to carry out this control two small samples (3 c.c.) of the medium are diluted in test-tubes with an equal bulk of distilled water. To the first tube are added 2 drops of 0·5 per cent. alcoholic solution of phenolphthalein, and to the second 2 drops of 0·02 per cent. watery solution of cresol-red to which 15 per cent. alcohol may be added as a preservative. If the reaction is correct, no colour change should occur in the first tube, while a rose to a pinkish colour should be noted in the second. Thus the reaction of the tested sample will not be as high as P_H 7·90, the point at which phenolphthalein first shows a trace of pink, but will be over 7·6, cresol-red developing a rose-pink (weak permanganate colour) at P_H 7·60. If this rose colour does not appear, but only a brown shade, the medium is on the acid side, while if a pink is obtained with phenolphthalein the medium is too alkaline. The error in either direction can be adjusted by the addition of a few drops of N/1 NaOH or HCl to the medium in bulk and the reaction again controlled.

Before use it is, of course, necessary to sterilise the medium by heat (autoclaving or steaming), but the increase in acidity at this stage is slight (less than P_H 0·2), and is sufficiently allowed for. The above procedure we have found quite satisfactory as an ordinary routine.

PROCEDURE RECOMMENDED FOR THE PREPARATION AND STANDARDISATION OF ORDINARY BROTH.

(1) Mince 1 lb. of meat free from fat; add 1000 c.c. of cold water. Boil for ¼ hour over a gas ring, filter through muslin and then through paper.

(2) Emulsify 10 grm. of peptone (any standard peptone such as Morson's or Witte's) in a mortar with some of the meat water. Then add the peptone together with 5 grm. of salt to the bulk of meat water.

(3) Steam for 20 minutes to dissolve the peptone completely; make up to 1000 c.c. The medium is now ready for standardisation.

(4) Measure 10 c.c. of the broth into a porcelain evaporating dish; add 25 c.c. of pure distilled water. Have a control dish alongside prepared in exactly the same manner. Add 5 drops of ½ per cent. alcoholic solution of thymolphthalein to the first dish. Then run in N/10 NaOH from a burette, stirring continuously, and looking for a darkening of the colour as compared with that of the control dish. Just before this point a precipitate of phosphates occurs. The point may be more aptly described as the disappearance of the yellow tint and the development of a bluish. Note the quantity of N/10 soda required to produce this change. In practice it is best to make four estimations, and the first in which a distinct blue has been produced is rejected. With the average data a calculation is made as to the amount of N/1 soda necessary to produce the same reaction in the rest of the broth. This amount of soda is then added and the flask shaken.

(5) The alkaline broth is now brought to the boil to deposit the phosphates, and then filtered free from them. The reaction is now adjusted by the addition of 10 c.c. of N/1 HCl per litre of broth. This will give a final reaction corresponding to that of plasma, or P_H 7·60.

(6) Control reaction with cresol-red and phenolphthalein.

(7) Lastly, place in tubes or flasks and sterilise in the autoclave for 20 minutes at 115° C.

Double-strength broth.—The double-strength broth indicated on the chart is prepared, except that all ingredients (meat, peptone, salt) are doubled, in a similar manner to that described above. Before use the medium is diluted with an equal volume of distilled water, tubed and sterilised.

DISCUSSION.

The aim of most recent workers upon the standardisation of media has been to provide a simple method which is well adapted for the routine use of a laboratory attendant. The hydrogen electrode is not, in our opinion, so adapted, and thus there has been a tendency towards colour indicators of H-ion concentration.

We ourselves find it most convenient to direct the attendant to titre out small samples of the medium with the help of a colour indicator, and to render the whole bulk "neutral" to our indicator by the addition of the calculated quantity of alkali. Having reached this point, he can add acid as indicated on a special "graph" for the medium in use to furnish a medium of any desired P_H. Finally he checks the reaction by the use of other dyes which indicate at different H-ion concentrations.

Other workers have developed practically the use of colour scales. These scales are prepared by the addition of the colour indicator to phosphate or phthalate solutions of which the H-ion concentration is known. These solutions, however, are much more difficult to prepare than is usually supposed, and to get them accurate they must be standardised against the hydrogen electrode.

To overcome this difficulty the Medical Research Committee have arranged for Messrs. Baird & Tatlock, 14, Cross St., Hatton Garden, London, E.C., to supply standard solutions of exact H-ion concentration (12). But even allowing that the standard solutions are of the correct concentration, there is no assurance that the same shade of colour will be given in the media as the standard solutions even when their reactions are identical, as it is well known that the presence of certain salts and albuminous materials can upset to a considerable extent the normal reactions of the indicator; in fact certain indicators (litmus, etc.) may be rendered almost useless. The colorimetric method recommended by Walpole (8), Cole and Onslow (13) and others only makes adjustment for the natural tint of the medium to be tested and not for the above phenomenon. Lastly, the non-permanence of the colour of the standard is a great objection.

Quite recently it has been claimed that very exact evaluations of the reaction of media can be made without the use of the elaborate solutions of standard hydrogen-ion concentrations. This method was recently introduced by Barnett and Chapman (14), and is based on the work of Salin. It consists in superimposing the extreme colours of a particular indicator. All that is necessary is weak acid and a weak alkaline solution. Barnett and Chapman advocated the use of phenol-red, which gives a range of reactions between P_H 7 and 8.

The test is carried out as follows: Two rows of six test-tubes are placed in a double rack, and into the tubes in the front row are measured 5 c.c. of dilute acid (1 drop of concentrated HCl in 100 c.c. of water) and into the other row 5 c.c. of dilute alkali (N/20 NaOH). A solution of the indicator having been prepared (phenol-red 0·01 per cent. watery solution), drops of it are added to the various test-tubes in the amounts indicated below.

Drops per Tube.

Series no.	Front row (acid series).	Back row (alkaline series).	P_H values.
1	9	1	6·9
2	8	2	7·2
3	7	3	7·5
4	6	4	7·7
5	5	5	7·9
6	4	6	8·1

To test media with this colour scale 1 c.c. is placed in a tube and diluted with 4 c.c. of distilled water. Then 10 drops of the indicator solution are added and the shade of colour compared with that of the above couples when viewed together. The reaction of the medium can be corrected by addition of weak acid or alkali, and a simple calculation will then give the amount necessary per litre. Although this procedure overcomes the difficulty of preparing complicated standard solutions, we have not found the method as accurate as has been stated. In the first place there is the old difficulty of matching shades; secondly, the scale colours do not completely match those of the colours in broth; and thirdly, the scale is not permanent. The reaction obtained was never nearer than 0·2 P_H as found by the hydrogen electrode when the scale was freshly made.

CONCLUSIONS.

(1) For ordinary routine purposes we recommend that the adjustment of the reaction of bacteriological media is best carried out by estimating the reaction by titration of a small volume, say 10 c.c., of the medium and calculating from the result obtained the alkali required to be added to the remainder. The final reaction is adjusted by the addition of N/1 HCl.

(2) For titration, thymolphthalein was found to be the most suitable indicator. This indicator changes colour at a point on the alkaline side of neutrality which ensures that sufficient phosphates will be thrown down to prevent their precipitation in the prepared medium.

(3) Very accurate and constant readings can be determined by means of thymolphthalein, as one is not dealing with the perception of a particular shade or colour, but with a definite and distinct change in the appearance of the medium.

(4) The most suitable end-point with thymolphthalein is the so-called neutral point. This corresponds to the first perceptible darkening observed with the reagent on the addition of alkali, the titration being carried out in the cold.

(5) The reaction of all bacteriological media should be expressed in hydrogen-ion concentration, preferably in Sörensen's nomenclature.

(6) For media we have adopted a reaction corresponding to that of plasma, namely P_H 7·6.

(7) All media should be titrated at room temperature.

(8) The final reaction of any particular batch of medium should always be controlled before it is issued for use.

(9) This control is most easily accomplished by the addition of certain colour indicators to small samples of the medium. In most instances the use of two indicators will give an estimation sufficiently accurate for ordinary use. In our hands cresol-red and phenolphthalein have proved sufficient.

(10) Three steamings for half an hour are never so destructive as one autoclaving at 115° C.

(11) Glucose and other sugar-containing media are especially easily hydrolysed and should never be autoclaved, only steamed.

(12) With certain reservations the reaction of media titrated by the chemical method can by means of a graph be expressed in H-ion concentration. Thus media of any required P_H values can readily be prepared.

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ON SYMPATHETIC FEVER AND HYPERPYREXIAL HEAT-STROKE.

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In the course of investigations on the function of the thyroid-adrenal apparatus as a humoral mechanism for the heat regulation of the body, observations have been made on the experimental fever produced by the injection of β-tetrahydronaphthylamine, which for the sake of brevity will be called in this paper, "T.H.N." The object of the present paper is to point out the similarity which exists between the experimental hyperpyrexia produced by T.H.N. and hyperpyrexial heat-stroke as it occurs in man.

The observations on T.H.N. fever were made mostly on mice and rats; some were made also on rabbits. There is an intense stimulation of the activity of the thyroid and adrenal glands which is demonstrable histologically. The effect has been described and illustrated in the recently published *Sixth Scientific Report of the Imperial Cancer Research Fund* (1), and need be referred to here only briefly. Adrenin is secreted into the blood-stream and there is a general stimulation of the sympathetic system. In fact, a rabbit under the influence of T.H.N. presents an external appearance almost identical with that of an unanæsthetised animal which has received a large dose of adrenin, the chief difference being that the effect of adrenin passes off in a few minutes and does not produce a pyrexia, while that of T.H.N. is prolonged. In both cases the blood-vessels of the ear are contracted, so that the ears feel cold; the palpebral fissure is widened and there is distinct proptosis, the pupil is dilated, although sometimes the dilatation is only slight, the respiration is rapid and forcible. In T.H.N. fever the anal sphincter is relaxed, so that the thermometer does not meet with any resistance when introduced into the rectum. Occasionally there is bleeding from the rectum. These appearances are somewhat different from those seen in an anæsthetised animal after an intravenous injection of a large dose of adrenin. In such an animal there is no obvious effect on the respiration and the widening of the palpebral fissure is not so obvious as the wide dilatation of the pupil. In rabbits a very characteristic effect of T.H.N.

is a general fine muscular tremor not unlike that of a rigor, and in addition occasional "stamping" of the hind legs due to a short forcible contraction of the leg-muscles. In mice and rats the most obvious effect of T.H.N. is the forcible and rapid respiration, the marked proptosis due to the widening of the palpebral fissure and the relaxation of the sphincter. The animals are excited and the fur is ruffled. The kymograph experiments of Jonescu (6) on anæsthetised animals refer to the immediate action of the substance itself, and show a stimulation of the sympathetic system partly by a central and partly by a peripheral action. This direct effect passes off quickly, and must not be confused with the later indirect effects which set in as the result of the prolonged activity of the suprarenal gland. Observations on metabolism (14) have shown a considerable and prolonged increase in the N-excretion, which is very marked on the day of administration and persists for the next two days when the temperature has become normal and then gradually falls again. It differs in this respect as well as in its general behaviour from the over-heated animal the temperature of which has been raised by the external application of heat. In such an animal there is no effect on the N-excretion on the day of the experiment; it rises the following day, falls rapidly and reaches the normal level again on the third day. There is no glycosuria in T.H.N. fever.

Of considerable interest in connection with the premonitory symptoms of hyperpyrexial heat-stroke in man are the effects on man of small doses of T.H.N. (14). All the doses given were so small that they did not produce a rise of temperature. Doses of 0·075 grm. to 0·1 grm. produced drowsiness, giddiness, headache, palpitations and a slight feeling of oppression. Sometimes the pupils were dilated, but not so much and not so frequently as in animals, and they continued to react to light. Slightly larger doses (0·15 grm. to 0·25 grm.) produced vomiting, violent headache, giddiness, and sometimes difficulty of breathing.

The rise in temperature begins as a rule within an hour after the subcutaneous injection of a suitable dose of T.H.N., and, in rats especially, is sometimes preceded by a fall in temperature. After the first hour the temperature continues to rise rapidly for the next two or three hours. Either the animal dies in hyperpyrexia, frequently in convulsions, or the temperature falls again rapidly in the fourth or fifth hour after the injection to the normal level. If the animal dies, rigor sets in within a few minutes. The fever is due, as Stern has shown (14), to increased heat-production and to diminished heat-loss. The mode of action by which this fever is produced has already been discussed in previous publications (1), and need only be summarised here. As a result of the increased activity of the thyroid and adrenal glands there is a mobilisation of the liver glycogen. In the absence of glycogen, carbohydrate is formed from proteins and possibly also from fats. It has been shown that the body reacts to the resulting influx of carbohydrates into the blood-stream under these conditions by an increased oxidation. Heat-production is thus increased. At the same time, heat-loss is diminished owing to the constriction of the cutaneous vessels. Since this type of fever can thus be explained as being produced by a stimulation of the sympathetic system, it may be suitably called "sympathetic fever."

The post-mortem examination of an animal which has died in T.H.N. hyperpyrexia shows as the most frequent and obvious feature an intense congestion of the lungs, which may be dark purple. Sometimes small hæmorrhages into the lungs occur which can be seen with the naked eye. The right ventricle is flaccid, the left ventricle is contracted. The trachea is often filled with mucus, the veins are engorged with dark blood. The thyroid and adrenal are deeply congested and so are the meninges. The appearance of the lungs again recalls the effect of adrenin which in toxic doses produces in unanæsthetised animals the same effect, and, in fact, often kills by hæmorrhage into the lung.

The production of hyperpyrexia by the injection of T.H.N. is dependent not only on the dose administered but also on some other factors, of which one is important for the purpose of the present paper. This factor is the climatic condition of the environment, in which the animal is kept after the injection, and the facilities it affords for cooling the animal. This can be demonstrated most clearly in the mouse, as the following experiments illustrate. Four mice were injected subcutaneously with 2·5 mgrm. T.H.N. Two mice were kept in a wooden cage with saw-dust, covered with a perforated lid in a warm room near the gas stove. The temperature inside the wooden cage was 30° C. The other two mice were kept in another room, temperature 16°, in glass jars without saw-dust, so that the mice were in contact with the relatively cold glass. Both the latter mice survived, and apart from showing a ruffled coat were not obviously affected by the T.H.N. The other two mice kept in the warm room passed into convulsions at the end of the first hour and died, the one at the end of the first hour showing post-mortem extensive hæmorrhages into the lung; the other mouse was found dead at the end of the second hour, the lungs also showing intense congestion. The weights and temperatures were as follows:

	Weight.	Before injection.	1 hour after.	2 hours after.
Mouse No. 1, hot room	17 grm.	35·1°	39·9°	Dead.
,, ,, 2, ,, ,,	22 grm.	36·4°	Dead 38·8°*	—
,, ,, 3, cool room	17·5 grm.	34·2°	35·5°	below 32°
,, ,, 4, ,, ,,	20·5 grm.	35·2°	33·4°	below 32°

* Taken immediately after death.

The importance of the condition of effective cooling in preventing or determining the onset of hyperpyrexia after the injection of T.H.N. can also be demonstrated in the following manner: A dose of 2·5–3 mgrm. T.H.N. is, as a rule, just sufficient to produce a fatal hyperpyrexia in mice of 15–20 grm. weight if kept in a warm room in separate cages. A dose of 0·5 or even 1 mgrm. produces, as a rule, no visible effect under these conditions. But if twelve mice which have received such a small dose are kept together in a cage many of them die within the first two hours with the typical symptoms of T.H.N. hyperpyrexia. The wooden cages which are used in this laboratory are of such dimensions that ten to twelve mice can be kept together in them comfortably, and that is, in fact, the way in which mice are kept in this laboratory for weeks or months. But when there occurs in the mice a stimulation of the sympathetic system, so slight that it produces neither visible symptoms nor a rise in temperature as long as each animal is kept in a separate cage and has thus good facilities for cooling, then the climatic conditions of the crowded cage elicit a fatal hyperpyrexia. To give an example of such an experiment: after an injection of 1 mgrm. T.H.N. six out of twelve mice died in one experiment. In another experiment in which four out of twelve mice died, the temperature of the crowded cage when the first mouse died was found to be 27° C.; in the control cage with two mice the temperature was 20°. The temperatures of four mice taken from the crowded cage two hours after the injection of T.H.N. were 42·6°, 42·0°, 40·8°, 39·5°. The temperatures of four isolated mice from control cages were 38·7°, 37·8°, 37·0°, 36·9°. Two isolated mice which had received double the dose of T.H.N. and survived, without having shown very marked symptoms at any time, reached 38·4° and 38·8°.

In rats the influence of cooling was demonstrated as follows: A rat, the coat of which had been clipped, was kept together with a normal control rat of the same age and weight in a cold room. After two days both rats received 0·02 grm. T.H.N. The temperature of the normal control rat, which before the injection was 37·1° C., rose to 40·0° C. in the first two hours; that of the clipped rat remained within half a degree of the temperature of 36·4° C. which it had before the injection.

Another interesting illustration of the relation between the temperature of the environment and the hyperpyrexia produced by sympathetic stimulation was obtained accidentally in the course of an experiment, the real object of which had been to study the effect of continued stimulation of the thyroid-adrenal apparatus by repeated injections of T.H.N. A rabbit, weighing 2400 grm., received during the last half of July, 1916, four injections of 0·06 grm. T.H.N. and five injections of 0·08 grm. T.H.N. on nine different days. On only two occasions did it show a slight pyrexia, the temperature rising from the normal temperature of 39·5° to a temperature of 40·7° and 40·8° respectively. It received the last of these injections on July 28th (0·08 grm. T.H.N.), but no pyrexia resulted. After three days it was again injected, on August 1st, with 0·07 grm. T.H.N., and died in three hours with a temperature of over 44°. At the time the importance of the outside temperature had not been recognised, and this result was looked upon as an unfortunate accident which could not be explained. On going through my protocols recently it occurred to me to consult the official meteorological records. They show that July, 1916, was an exceptionally cool month. From July 11th, 1916, to July 26th the maximal temperature ranged between 61° F. and 76° F. On July 27th the weather improved and the temperature rose to 78° F., and hot weather set in during the last two days of July and the first day of August. The connection between this change in the weather and the effect of T.H.N. appears from the following table:

Date.	Official max. temp.	Injection of T.H.N.	Result.
26/7/'16	73° F.	0·08 grm.	No fever.
27/7/'16	78° F.	,,	,,
28/7/'16	78° F.	,,	,,
29/7/'16	79° F.	No injection	—
30/7/'16	82° F.	,,	—
31/7/'16	84° F.	,,	—
1/8/'16	83° F.	0·07 grm.	Dead in 3 hours. 44°.

It may be added that the interval of three days without injections cannot be made responsible for this result, since in the preceding period such intervals had occurred; the resumption of the injections had not always resulted in fever.

Another point which may be mentioned is, that in this experiment the characteristic muscular contractions described above as "stamping," and the other symptoms of sympathetic stimulation (widening of the palpebral fissure, contractions of the blood-vessels of the ear), appeared after the injection of T.H.N. even when there was no fever. This was also observed in experiments in other rabbits. These effects are not, therefore, the results of a high body temperature acting as a stimulus to nerve or muscle.

It should be noted that besides this factor of outside temperature and of cooling, there are yet other less well understood conditions which determine the onset of sympathetic fever after the administration of T.H.N. in animals, especially in rabbits. Many rabbits fail to give any rise of temperature after doses which will kill other rabbits in hyperpyrexia, although both are kept in the same room. On going into this curious fact, it was found that rabbits which had been kept for two months or more in the laboratory, and which were well fed, sleek, and in good condition, never failed to respond with fever to a dose of 0·04 grm. T.H.N. per kilo. But the same dose would not produce any effect on rabbits which had just been brought in and which were in a much poorer condition. In rats, intercurrent diseases, such as pneumonia or a septic bronchiectasis, frequently interfered with the typical response to the administration of T.H.N. Animals so affected died, as a rule, after the injection of T.H.N., but without having developed a rise in temperature. Sometimes, however, the animals do not die, nor do they show a rise in temperature, and no obvious cause for the failure to respond to T.H.N. could be found.

From the experiments detailed above on the importance of the outside temperature and the condition of cooling in determining the effect produced by T.H.N., it is clear that these external conditions governing the heat loss are the chief factors determining whether prolonged stimulation of the sympathetic system leads to hyperpyrexia or not. This fact may be expressed as follows: A stimulation of the sympathetic system, prolonged, but so slight that under certain conditions no obvious symptoms are produced, may be turned into a rapidly fatal hyperpyrexia by conditions which lead to an interference with the heat loss—an interference so slight that it has no ill-effect on a normal animal.

Formulated in this way the T.H.N. hyperpyrexia acquires the features of an "experimental heat-stroke" and its resemblance to hyperpyrexial heat-stroke in man becomes apparent. This resemblance extends to the post-mortem findings in hyperpyrexial heat-stroke in man and those of experimental T.H.N. heat-stroke in animals. There is early rigor, and all authors lay stress on the intense congestion of the lungs without consolidation. Manson (8) speaks of the "phenomenally engorged lungs." The venous system generally is engorged; according to the statement by Wood in Pepper's *System of Medicine* (10), "blood appears to leave the arterial system and collect in the venous trunks." Hearne (4) has found œdema and hyperæmia of the cerebral membranes, and in the brain evidence of hæmorrhages into the perivascular spaces of the larger arteries, and Rogers (13) mentions the occurrence of petechial hæmorrhages in brain, viscera and skin. All these facts agree with the postulate of the occurrence of sympathetic stimulation with the resultant high blood-pressure. Histological examination of the thyroid and adrenal glands are likely to yield further valuable and interesting information on this point. Apparently such an examination has never been carried out.

In drawing a comparison between disturbances of the heat regulation in man and in animals, it is necessary to bear in mind an important difference which exists in the physical mechanism of heat regulation. In man this is carried out largely by the activities of the sweat-glands. In most animals—the horse being one of the few exceptions—the activity of the sweat-glands is of no practical importance as a means of regulating the body temperature, since the distribution of these glands is limited to the hairless pads of the feet. For the activity of the sweat-glands there is then substituted another physical mechanism, also under the control of the sympathetic, namely, the erection of the hairs of the fur, which also increases the heat-loss.

This difference in the physical mechanism of heat regulation is of importance in the present instance, because one of the characteristic features of true hyperpyrexial heat-stroke in man is the suppression of sweating. How this is brought about is not clear. Hearne (3, 4) attributes it to an exhaustion of the sweat-glands. It is difficult to understand why such an exhaustion should occur, since the secretion of sweat does not involve the formation of a specific product of secretion by the gland-cells, but is simply the secretion of water in which about 0·5 per cent. of sodium chloride and a trace of urea are dissolved. But there is evidence that the activity of the sweat-glands is under the influence of inhibiting factors. Pembrey (9) points to the familiar evidence of the suppression of sweating at the onset of many fevers. He suggests that this is due to a paralysis of the secretory mechanism of the glands produced by the circulating toxins, and that in heat-stroke some unusual product of metabolism acts in a similar way. Several authors have claimed to have demonstrated the existence of inhibitory nerve-fibres to the sweat-glands (11). Of most interest in this connection is an old observation of Luchsinger's (6)—that the external application of heat may inhibit the activity of the sweat-glands. Thus, if on a warm day one hand be held for ten minutes in water at 45° to 50° C., while the other is immersed in water at 15° to 30° C., and exercise is then taken, the hand which was in water at the lower temperature commences to sweat at once, the other not for some considerable time. Experiments on the effect of T.H.N., under different conditions of the temperature of the environment, on animals capable of regulating their heat-loss by sweating might give further information on this point.

Whichever explanation may be found to be correct, the suppression of sweating in heat-stroke is of importance because it is in itself sufficient to bring about a rise of body-temperature, as the observations of Tendlau (16) showed. These were made on an individual who suffered from a congenital deficiency of sweat-glands, and whose temperature on doing heavy muscular work or on merely sitting in the sun would rise to 40° and 41° C. But the condition of hyperpyrexial heat-stroke comprises more than a rise in the body-temperature. There is an early stage which points to a general stimulation, and the symptoms of which are very similar to those of sympathetic stimulation produced in man by small doses of T.H.N. So far as one can judge from the clinical data available, this condition occurs at ranges of the body temperature which do not produce a similar effect in normal men whose temperature has been artificially raised by being exposed to a moist warm atmosphere. The difference is the same as that existing between a rabbit suffering from T.H.N. fever and a rabbit the body-temperature of which has been raised by over-heating. As Stern (13) points out, the latter lies stretched out in the cage with dilated cutaneous vessels and relaxed muscles, avoiding every movement. The former is restless, excited, "stamps," and its cutaneous vessels are constricted. The rise in body-temperature is not therefore in itself an adequate explanation of all the features of hyperpyrexial heat-stroke, and from this point of view, too, it is necessary to assume the existence of an intrinsic factor.

The conception of hyperpyrexial heat-stroke presented in this paper is, then, that it is due to the combination of an extrinsic factor, namely the deficient cooling due to the conditions of the environment, and an intrinsic factor, namely the hyperactivity of the sympathetic system resulting from the overactivity of the thyroid-adrenal apparatus. This conception recalls the explanation of hyperpyrexial heat-stroke given by van Gieson, Ewing and others (9), who postulated the existence of an autogenetic poison. The question now remains how this overactivity of the thyroid-adrenal apparatus is elicited. An explanation may be found from a consideration of the limitations of the thyroid adrenal apparatus as a heat-regulating mechanism. It is very efficient as long as the temperature of the body has to be kept on a level considerably higher than that of the environment. For not only can its activity increase the heat-production, it can also, if sufficiently great, diminish the heat-loss by the constriction of the cutaneous blood-vessels. This inhibits the activity of the sweat-glands, which might otherwise occur and increase the heat-loss. But as the rate of cooling diminishes with a rise of the temperature of the environment, or with a change in the other factors which determine it, as Leonard Hill (5) has shown, the thyroid-adrenal apparatus has to contend with an increasingly difficult problem. It cannot by its activity diminish the heat-production, and it could only increase the heat-loss if it could stimulate the sweat-glands alone, without at the same time producing the other effects of sympathetic stimulation. Under these circumstances the slightest activity of the sympathetic system would lead to fatal results. There must exist, therefore, a mechanism which is capable of restraining the activity of the thyroid-adrenal apparatus, and the relaxing effect of a hot climate, and especially a hot moist climate, may be looked upon as the psychological expression of such a damping mechanism. Whether this restraint is produced by an actual inhibition of the secretion of adrenin, or by an action on the sympathetic nerve-endings which renders them less sensitive, or in yet another way, is not known. It may be pointed out in passing that there are other facts which demand the assumption of a restraining mechanism as an explanation. Since the adrenal medulla secretes adrenin as the result of sympathetic stimulation, it should do so also as the result of the presence in the blood of adrenin. If that were so the secretion of adrenin by the gland, when once initiated, should lead to a continued secretion of adrenin which should go on until the load of the gland is exhausted. We know, however, that this does not occur—at any rate in the normal animal. In hyperpyrexial heat-stroke this restraining mechanism breaks down and such a vicious circle is initiated. This conclusion is in agreement with the results obtained by Sutton (14) in his experiments made on himself on the effect of a hot moist atmosphere on the gaseous metabolism and the temperature of the normal human organism, and affords an explanation of the effects observed by him. Sutton states that, "once the balance of the mechanism of heat-regulation has been definitely upset by high external temperature combined with almost total abolition of heat-loss in evaporation a vicious circle is established. The internal temperature rises, and as a result the oxidation processes and therefore the production of heat also increases, so that the body-temperature rises still further, and so on. Once the ball has been set rolling nothing seems to check its progress, and it slowly but surely gathers speed." Very significant, from the point of view developed in this paper, is the rise of the respiratory quotient, observed by Sutton, from 0·77 at the beginning of the experiment to 0·916 at the end, which indicates according to his calculation an increase of carbohydrate consumption to at least five to six times its previous amount. From what we now know of the relation of the activity of the thyroid and adrenal glands to carbohydrate metabolism, which has been briefly summarised at the outset of this paper, this increased oxidation of carbohydrates indicates a hyperactivity of these glands. The vicious circle referred to by Sutton is this hyperactivity of the thyroid-adrenal apparatus which we postulated as the intrinsic factor in hyperpyrexial heat-stroke.

The view put forward on the nature of heat-stroke has an obvious bearing on the treatment of the condition. The paramount importance of reducing the body-temperature by drastic cooling is obvious and generally recognised. But this treatment is, as a rule, instituted only when the condition of heat-stroke has fully declared itself, and the temperature has risen considerably. In that case this treatment only combats the symptoms and is accordingly not very successful. But, as Hearne points out (3, 4), hyperpyrexial heat-stroke gives ample warning of its onset (according to him from one to forty-eight hours), and he contends that the onset of heat-stroke can with certainty be completely aborted if treatment is instituted in this prodromal stage. Rogers also emphasises the importance of an early and prophylactic treatment. The views put forward in this paper support Hearne's contention and so do the experiments. We refer here particularly to the experiments in which mice, after a given dose of T.H.N., developed experimental heat-stroke when kept in a crowded cage, but showed no ill-effects, and not even a rise in temperature, if kept under conditions admitting of efficient cooling.

In addition to reducing the temperature, there are accessory methods of treatment which may be discussed.

The injection of saline or other fluids has been recommended. That, however, is clearly contra-indicated in this condition. Venesection, which has also been recommended, is not open to the same objection, but it is not clear how it can combat the condition beyond relieving the symptoms. Morphine has been recommended as a sedative. This drug acts differently in different species of animals, so that in this case deductions from observations on animals may not necessarily be valid for man. Still it is important to note that in mice the injection of morphia, together with T.H.N., did not reduce the mortality when compared with animals which had received the same dose of T.H.N. alone under the same conditions. On the contrary, so far as the experimental evidence goes, it shows that after morphine the mortality is higher and the animals die sooner, as the following record shows:

Experiment.	No. of mice.	Dose of morphine in mgrm.	Dose of T.H.N. in mgrm.	Result.
I	3	4 mgrm.	3 mgrm.	After 2½ hrs. all three dead.
	2	—	3 ,,	After 2½ hrs. both mice alive, but one very ill. Dead after 3 hrs. One mouse survived.
II	3	0·5 mgrm.	2·5 ,,	After 2 hrs. all three mice dead.
	3	—	2·5 ,,	After 2 hrs. one dead, two ill. Two mice survived.
III	4	3 mgrm.	2·5 ,,	After 2 hrs. three mice dead. The fourth mouse died half-an-hour later.
	4	—	2·5 ,,	After 2 hrs. one mouse died; two others died within the next half hour. One mouse survived.
	4	3 mgrm.	—	All four mice alive and well throughout.

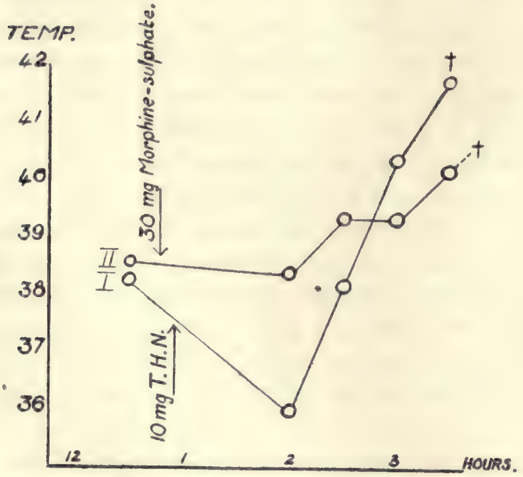

Fig. 1.—Effect of morphine on T.H.N. fever in rats. Curve 1: Temperature of control animal. Curve 2: Temperature of morphinised animal. Both received 10 mgrm. T.H.N. at the same time. † indicates death of the animal.

Morphine modifies the action of T.H.N. so that the temperature rises without being accompanied by an excitement of the animal. The symptoms are thus less violent, and the appearance of a beneficial effect may be simulated in man and may have led to its use. But from the experimental evidence its use is strongly contra-indicated. The effect of morphine in the temperature curve of T.H.N. hyperpyrexia is given in Fig. 1.

For the treatment of experimental T.H.N. heat-stroke chloral is a much more suitable drug. In mice and rats I have found it possible to delay the onset of experimental T.H.N. heat-stroke by doses of chloral which will produce a slight anæsthesia if given alone, and even to abort it completely. The results of three such experiments on rats are given in Figs. 2 and 3. There appears to be a physiological antagonism between chloral and T.H.N.

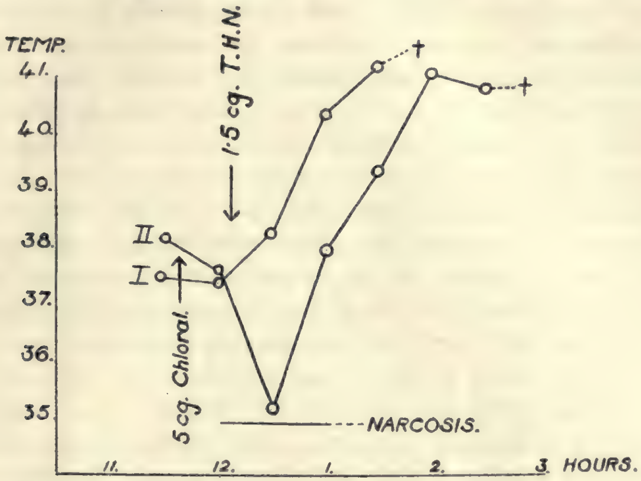

Fig. 2.—Effect of chloral on T.H.N. fever in rats. Curve 1: Temperature of control rat. Curve 2: Temperature of chloralised rat. Both animals received 15 mgrm. T.H.N. at the same time. The period during which chloral exercised its narcotic effect is indicated by the horizontal line marked "narcosis."

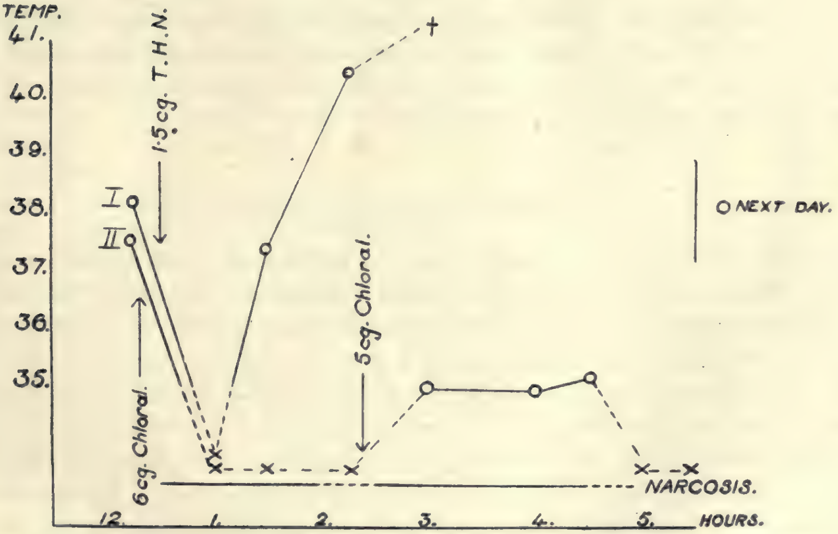

Fig. 3.—Prolonged effect of two doses of chloral on T.H.N. fever in rats. Curve 1: Temperature of control rat. Curve 2: Temperature of chloralised rat. The thermometer did not register temperatures below 35°C. Such observations are indicated by the sign ×.

So long as the narcotic effect of chloral predominates the animal is asleep and shows none of the effects of T.H.N. which are visible in the non-chloralised animal; the temperature remains sub-normal, even after the injection of a dose of T.H.N., which proves fatal to a normal animal. On the other hand, the effect of chloral does not last as long in an animal which has received T.H.N. as it does in a normal animal, and as soon as the narcotic effect of chloral passes off the temperature begins to rise, and the animal may still die in hyperpyrexia (see Fig. 2). But if the narcotic effect of chloral is maintained by means of a second dose, the experimental heat-stroke may be completely aborted (Fig. 3). In the experiment represented by Fig. 3 the prolonged effect of chloral prevented the temperature from rising, and the animal was alive and well for three months after the experiment.

The effect of chloral is probably not a specific one, but due to the condition of anæsthesia produced by it. For there is experimental evidence that in anæsthesia the effect of a given dose of adrenin is altered and less forcible than in a normal animal. And Fawcett and Hale White (2) found that rabbits in ether anæsthesia not only failed to respond to the injection of T.H.N. with a rise of temperature, but that this drug was not even able to prevent or reduce the fall of temperature produced by the anæsthetic. The significant difference between the action of morphine and chloral lies in the fact that morphine, while exercising its characteristic effect in subduing the excitement of the animal, fails to prevent the rise in body-temperature and the death of the animal, and perhaps even accelerates the lethal issue. Chloral, on the other hand, as long as its effect lasts, suppresses not only the visible symptoms of T.H.N., but also the fever.

SUMMARY AND CONCLUSIONS.

By the injection of suitable doses of β-tetrahydronaphthylamine a prolonged sympathetic stimulation associated with an over-activity of the thyroid-adrenal apparatus is produced. The increased heat-production and diminished heat-loss resulting therefrom induces a pyrexia, which from its origin it is proposed to designate as "sympathetic fever." The height of the pyrexia is chiefly dependent upon the climatic conditions of the environment. Exposure to a cool environment, by increasing the heat-loss, diminishes the height of the fever or may prevent the body-temperature from rising. An interference with the rate of cooling by exposure to a warm atmosphere or by keeping animals crowded together elicits a rapidly fatal hyperpyrexia. This hyperpyrexia, from the post-mortem findings and the conditions under which it occurs, presents such a striking resemblance to hyperpyrexial heat-stroke as it occurs in man that it may be described as an "experimental heat-stroke." This resemblance is confirmed by a comparison of the premonitory symptoms of hyperpyrexial heat-stroke in man and the effect in man of very small doses of β-tetrahydronaphthylamine. The explanation of hyperpyrexial heat-stroke in man is, therefore, that an extrinsic factor, namely, deficient cooling due to the conditions of the environment, acts upon an organism which is under the influence of prolonged sympathetic stimulation due to an excessive activity of the thyroid-adrenal apparatus.

The bearing of this view on the treatment of heat-stroke is discussed.

It gives me great pleasure to acknowledge my indebtedness to Dr. K. G. Hearne for much valuable information on hyperpyrexial heat-stroke in man derived from his extensive experience of this condition as it occurred in Mesopotamia among the troops.

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THE SEROLOGICAL CLASSIFICATION OF MENINGOCOCCI.

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SEROLOGICAL differences among meningococci were first clearly established by Dopter (1). He showed that cocci might be isolated from the cerebro-spinal fluid which did not agglutinate with meningococcus serum, although they agglutinated with a serum made from themselves. He called these strains "parameningococci," and later subdivided these into further serological sub-groups.

Arkwright (2) also, in this country, found serological differences among meningococci, and was able to divide his strains into two main groups, which, however, were not distinct, but merged one into the other.

At the beginning of the war Gordon and Murray (3) examined a large number of cultures of meningococci which were obtained from cases of "epidemic" cerebro-spinal fever in the Army. By preparing agglutinating sera from single strains they found that four different monovalent sera were sufficient to agglutinate all specimens, and on this basis divided them into four "types."

In numerous later papers Gordon (4) has held that his four types are serologically distinct, as shown by the sharp result of absorption tests, and that practically all strains from "epidemic" cases could be relegated to one of his "types."

The "distinctness" of the serological types was disputed by Griffith and Scott (5), working under the late Local Government Board upon civil cases. By using monovalent sera they were only able to distinguish two main groups, which, however, overlapped, and they held that a serological classification such as Gordon's depended for its sharpness upon an arbitrary selection of standards for the different "types."

The attitude of Gordon was largely supported by Fildes and Baker (6), who, working upon the "epidemic" of 1916–17 in the Navy, agreed that all (except one) strains of meningococci obtained from the cerebro-spinal fluid were capable of being referred to one or other of Gordon's four types. With regard to the criticisms of Griffith and Scott, they admitted that the serological relationship between types I and III on the one hand and types II and IV on the other were such that confusion between them might arise from the selection of arbitrary standards, but held that the group I–III differed so markedly from the group II–IV that no arbitrary selection of standards would lead to confusion between them. They considered that two main serological groups of meningococci existed, and that each group contained two outstanding serological characters which might be combined together in one bacterium, and thus produce "intermediate" serological varieties.

Gordon himself (4) admitted that the sporadic civil cases of Griffith and Scott might show a less sharp serological differentiation than "epidemic" cases, but denied that in these latter there was any relationship between the types so close that they were capable of being confused by an arbitrary selection of standards.

A study of a large number of epidemic military strains was made by Tulloch (6), and he was able to place 101 out of 107 strains into one or other of Gordon's types. He found, however, that type II cases could not always be so identified upon investigation with one standard only, but he required to select three strains of standards before this could be achieved.

It appeared, therefore, that even among military strains there were some which could not be identified by Gordon's methods, while type II at least could not be looked upon as a uniform and definite variety.

The investigation of cases in the Navy was continued during the period 1917–18. The number of cases during that winter was smaller than in the previous winter and thus they might be described as "sporadic," but there was not really any essential difference in the curve of incidence. It was found that the number of cases which could not be classified as Gordon's types was larger, and the present paper represents a study of the value of "type" classification upon the basis of these cases.

It was decided that the whole question of selection of standards must be avoided and that the absorptive power of one strain must be tested with a serum which "offered" agglutinins of all shades of type, instead of with a so-called "monotypical" serum, which was not "monotypical" at all. Such a serum, capable of agglutinating all types of meningococci, when absorbed with strain A would lose all agglutinins for strain A, and if any other strains (B, C, D) were similarly affected they might be classed in the same group; but it must be shown that absorption with B, C or D did not upset this grouping before its correctness was accepted.

The serum which was selected was a horse-serum prepared for treatment purposes, and this has a particular value for the present experiment, because the agglutinin response of the horse to an injection of a meningococcus is very broad and polyvalent. In a young rabbit the first agglutinins to appear are more "specific" to a particular strain, while in the horse the repeated inoculation of one strain will produce agglutinins for all types of meningococci. This is well shown in a test of the monovalent meningococcus serum prepared for the Medical Research Committee at Cambridge. Tested in June, 1918, each "type" serum agglutinated all "type" cocci up to a dilution of 1 in 400 to 800.

The selection of a horse-serum, therefore, ensured that agglutinins for all strains of meningococci would be present, which was necessary for the success of the experiment.

A collection of meningococci from the cerebro-spinal fluid was made and each of these strains was used to absorb the polyvalent anti-meningococcus serum. The serum was shown to exert an agglutinating effect upon all, and this action was again tested after the agglutinins for each strain separately had been removed. In this matter it was shown that absorption with one strain removed the agglutinins for itself and for certain others, and such could then be grouped together as being of similar serological character. Such grouping did not depend in any way upon arbitrary selection of standards.

Twenty-one different strains of meningococci were tested. Each was grown upon a hæmoglobin-serum-agar medium, and thick suspensions were made of each. These suspensions were diluted with carbolic saline to an opacity corresponding to 8000 million cocci per c.c. and kept as stock.

The polyvalent serum was obtained from the Rockefeller Institute and was dated July 25th, 1917, the experiment being carried out in January, 1918. The serum was diluted in bulk to 1 in 50 and the same serum and suspensions were used throughout the whole experiment.

Equal parts of the diluted serum and stock suspension were mixed together and kept in the ice chest for eight hours and then in the incubator for eight hours. The tubes were thoroughly centrifuged and the supernatant fluid taken off. This serum, now diluted 1 in 100, was then used to agglutinate all strains of cocci, the stock suspensions being diluted 1 in 4 for this purpose. The ultimate dilutions of serum were 1 in 200, 1 in 400, 1 in 800, and 1 in 1600. The unabsorbed serum was also put up with the same suspensions of cocci in the same dilutions, and the results with the absorbed serum were compared with those of the unabsorbed.

The following is an example of such an experiment :

Flexner Serum absorbed with Strain St., Ds., and Hr. separately and tested upon 21 strains.

Strains on which the absorbed serum was tested.	Serum absorbed with strain.												Not absorbed.			
	St. 1 in				Ds. 1 in				Hr. 1 in				1 in			
	200	400	800	1600	200	400	800	1600	200	400	800	1600	200	400	800	1600
St.	0	0	0	0	0	0	0	0	2	2	2	2	2	2	2	1
Ds.	0	0	0	0	0	0	0	0	2	2	2	1	2	2	2	2
Hg.	1	0	0	0	0	0	0	0	2	2	2	1	2	2	2	2
Re.	1	0	0	0	2	1	0	0	2	2	2	0	2	2	2	1
An.	0	0	0	0	0	0	0	0	2	2	2	1	2	2	2	1
Hr.	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0
Js.	0	0	0	0	0	0	0	0	2	2	1	0	2	2	1	0
Le.	0	0	0	0	0	0	0	0	2	0	0	0	1	1	0	0
Tr.	2	2	2	2	2	0	0	0	2	2	2	2	2	2	2	2
Ck.	2	2	2	1	2	2	2	1	2	2	2	1	2	2	2	1
Ws.	2	1	1	0	2	1	0	0	2	0	0	0	2	2	0	0
Cr.	2	2	2	1	2	2	2	1	2	2	2	1	2	2	2	2
Hn.	2	2	2	1	2	2	2	1	2	2	2	1	2	2	2	1
Fr.	2	2	2	0	2	2	2	0	2	2	2	0	2	2	2	0
Kl.	2	2	2	0	2	2	2	1	2	2	2	1	2	2	2	1
Ce.	2	1	0	0	1	1	0	0	2	1	0	0	2	1	0	0
Ct.	2	2	2	1	2	2	1	1	2	2	2	1	2	2	2	1
Br.	2	2	2	1	2	2	2	2	2	2	2	0	2	2	2	1
Hs.	2	2	2	2	2	2	2	2	2	2	2	1	2	2	2	2
P.	2	2	2	0	2	2	2	0	2	2	1	0	2	2	2	0
Bn.	0	0	0	0	0	0	0	0	0	0	0	0	2	2	1	0

2 = complete agglutination with the dilution indicated.
1 = incomplete agglutination with the dilution indicated.
0 = no agglutination with the dilution indicated.

From these figures the degree of absorption was calculated in the following way:

When, upon comparison of the agglutinating power of the absorbed serum with that of the unabsorbed, it was found that no removal of agglutinins had taken place or that less than half of the full content of agglutinins had been removed, the result is marked as 0 against the strain whose agglutinins were under question; thus in the following table no Tr. agglutinins were removed by absorption with St. When half of the agglutinins were removed the sign + is used, and when over half, the sign +.

Strains on which the absorbed serum was tested.	Serum absorbed with strain— St.	Ds.	Hr.
St.	+	+	0
Ds.	+	+	0
Hg.	+	+	0
Re.	+	+	0
An.	+	+	0
Hr.	+	+	+
Js.	+	+	0
Le.	?	?	?
Tr.	0	+	0
Ck.	0	0	0
Ws.	0	0	+
Cr.	0	0	0
Hn.	0	0	0
Fr.	0	0	0
Kl.	0	0	0
Ce.	0	0	0
Ct.	0	0	0
Br.	0	0	0
Hs.	0	0	0
P	0	0	0
Bn.	+	+	+

Note.—The agglutinability of the particular suspension of strain Le. used was so slight that no inferences could be drawn.

From this table it may be deduced provisionally that Hr. in the serological sense is similar to Bn., because the agglutinins for Bn. are removed by absorption with Hr.; and that St. is not identical to Ds., because absorption with Ds. removes agglutinins for Tr., while absorption with St. does not. The full significance of the grouping cannot, however, be apparent until a larger number of strains have been tested.

In the next table the whole twenty-one strains are shown, and the extent of the absorption from Flexner serum noted in each case. These figures were arrived at by carrying out two or three absorption experiments with the unabsorbed control daily until the series was complete. The same stock materials were used throughout.

The cases are rearranged in order to show the serological groups into which they fall. Strains which absorb similar or almost similar agglutinins are grouped together.

Cocci Used for Absorbing Flexner Serum.

Cocci upon which the Absorbed Serum was Tested.

	Tr.	Hg.	Ds.	An.	Js.	Le.	St.	Re.	Hr.	Bn.	Kl.	Ce.	Ws.	Cr.	Ct.	Ck.	Hn.	Hs.	Fr.	P.	Br.
Tr.	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hg.	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ds.	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0
An.	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0
Js.	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0
Le.	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
St.	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0
Re.	+	+	+	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0
Hr.	+	+	+	+	+	0	+	+	+	+	+	+	0	0	0	0	0	0	0	+	0
Bn.	+	+	+	+	+	+	+	+	+	+	+	+	0	+	0	0	0	0	0	0	0
Kl.	0	0	0	0	0	0	0	0	0	0	+	+	0	0	0	0	0	0	0	0	0
Ce.	0	0	0	0	0	0	0	0	0	0	+	+	0	0	0	0	0	0	0	0	0
Ws.	0	0	0	0	0	0	0	0	+	0	0	0	+	+	+	+	+	0	0	0	0
Cr.	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	0	0	0
Ct.	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	0	0	0
Ck.	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	0	0	0	0
Hn.	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+	0	0
Hs.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	+	+	+	0
Fr.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	0
P.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	+	+	+	0
Br.	0	0	0	0	0	0	0	0	0	0	+	+	0	0	0	+	0	+	0	+	+

It will be observed that most of the strains are capable of being arranged into four groups, which are shown enclosed in squares. They may be referred to as groups A, B, C, D, from above downwards. In the main, the absorption by one strain is confined to the agglutinins for the other members of the same group. There is very little absorption in the portions of the table outside the groups, except where groups A and B abut, and where groups C and D abut. Group A, consisting of eight members, is connected with the small group B by the intermediate group containing strains Hr. and Bn. These two bacteria absorb agglutinins for themselves only, but both the large group A and the small group B absorb the agglutinins for Hr. and Bn. in addition to their own agglutinins. Thus these two bacteria must be looked upon as intermediate forms between the more distinct members of groups A and B. In fact, groups A and B and the intermediate forms may be looked upon as one "super-group."

Similarly group C, containing five members, is related to group D containing four, as shown by the slight degrees of absorption in the neighbourhood of their junction.

From this experiment, therefore, it appears justifiable to assume that there are two practically distinct serological groups among meningococci, and that each group consists of bacteria with two main serological characters, and also bacteria with these characters merged into one another to a greater or less extent.

In order to determine the relation of these groups to Gordon's types, cultures of his standard cocci, with which he had supplied me, were introduced into the experiment.

The strain Le. was Gordon's type I standard. Ce. was his type III standard. Hn. was his type II standard, although later he supplied me with Fr. as type II. Fr., however, in our classification falls into another group (type IV). Hs. and P. were both his type IV strains. With regard to Ct., this strain was used by me as a type IV standard on the strength of its reaction with type IV serum supplied to me by Col. Gordon, but it is now shown to be in a different group to Gordon's type IV strains and in the same group as his type II strains.

Thus it may be said that Gordon's types I and III correspond to the main characters of super-group I, but they do not account for the intermediate strains Hr. and Bn. Similarly his types II and IV correspond to the main features of super-group 2, but his classification does not suggest the marked degree of relationship between his two types. In fact, there is evidence of some confusion of standards owing to the closeness of relationship, which leads to type IV cocci being called type II, and type II cocci being called type IV.

Thus it will be seen that the grouping into Gordon's four types does not depend upon a selection of standards, since it emerges from this experiment in which no standards exist. But at the same time the relationship between types II and IV is so close that the change of a standard culture may even lead to a change in the "type" of the organism, and in this sense the selection of standards for sub-group characters is arbitrary and may lead to confusion. Further, even among epidemic cocci (Naval), strains may occur commonly which do not belong distinctly to any of Gordon's types, but fall between them.

The existence of intermediate strains is important because they correspond closely with cocci which are often isolated from the naso-pharynx and discarded as not meningococci because they do not agglutinate with "type" serum nor absorb "type" agglutinins. The "intermediate" cocci behave in the same way as shown in the following tables:

The Effect of "Type" Serum upon "Intermediate" Cocci.

Suspension of strains.		Agglutinating serum. Type I (Hg.). 1 in				Type III (Ce.). 1 in			
		100	200	400	800	100	200	400	800
An.	Type I	2	2	2	2	2	1	0	0
Re.	,,	2	2	2	2	2	1	0	0
Js.	,,	2	2	2	0	0	0	0	0
Hg.	,,	2	2	2	2	1	1	0	0
Cl.	,,	2	2	1	0	0	0	0	0
Le.	,,	2	2	2	1	1	0	0	0
Ds.	,,	2	2	2	2	2	1	0	0
St.	,,	2	2	2	2	2	1	0	0
Cn.	Type I–III	1	0	0	0	1	0	0	0
Bn.	,,	1	0	0	0	1	0	0	0
Hr.	,,	1	0	0	0	1	0	0	0
Kl.	Type III	2	1	0	0	2	2	2	1
Ce.	,,	0	0	0	0	2	1	0	0

In this experiment it is seen that type I and III sera have usually an agglutinating action on both types, but each agglutinates its own type to a higher dilution. The three intermediate cocci are hardly agglutinated by either serum.

The Effect of Type Serum after Absorption with "Intermediate" Cocci.

	1 in 100	200	400	800
Type I serum (Hg.) absorbed with Cn. and tested on type I (Le.)	2	2	2	0
,, ,, ,, Bn. ,, ,,	2	2	2	0
,, ,, ,, Hr. ,, ,,	2	2	2	0
,, ,, ,, An. (type I) ,, ,,	1	0	0	0
,, ,, ,, Ce. (type III) ,, ,,	2	2	2	0
,, ,, unabsorbed ,, ,,	2	2	2	0
Type III serum (Ce.) absorbed with Cn. and tested on type III (Kl.)	2	2	2	0
,, ,, ,, Bn. ,, ,,	2	2	2	0
,, ,, ,, Hr. ,, ,,	2	2	2	0
,, ,, ,, An. (type I) ,, ,,	2	2	2	0
,, ,, ,, Ce. (type III) ,, ,,	0	0	0	0
,, ,, unabsorbed ,, ,,	2	2	2	0

Thus the intermediate cocci neither remove type agglutinins from the sera nor are they obviously agglutinated by the type sera. They are, however, like the naso-pharyngeal cocci agglutinated by Flexner serum.

From these results it is clear that these intermediate cocci, if found in the throat, would be classed as "non-epidemic" if the type sera are relied upon for diagnosis, and this is one of the great technical fallacies in trying to rid a population of "carriers."

The term "non-epidemic" has become practically synonymous with "non-pathogenic." This was due to the fact that Gordon (and Fildes and Baker) found very few cocci in the cerebro-spinal fluid when the epidemic was more wide-spread, which did not conform to Gordon's types. Thus it was held that cocci from the naso-pharynx which did not conform to Gordon's types might be overlooked as harmless.

This was probably quite a justifiable assumption to make under the then prevailing circumstances, but it clearly has little scientific foundation, and if agglutination by four mono-typical sera is taken as the basis for the diagnosis of meningococci for the future, serious error (in the bacteriological sense) is liable to be introduced.

CONCLUSIONS.

(1) Meningococci may be divided into two distinct serological groups. Each group is capable of being analysed into two main serological sub-groups, and all cocci in the main group display each of these sub-characters to a greater or less extent.

(2) This classification is not dependent upon an arbitrary selection of standards.

(3) A selection of standards for closely-defining sub-group characters is an arbitrary proceeding, and may lead to confusion if one and the same strain is not maintained as standard.

(4) The use of monovalent sera manufactured from arbitrary standards of sub-group characters will fail to reveal certain cocci as meningococci, although these may have been isolated from the cerebro-spinal fluid of a case of the disease.

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ON THE TESTING OF RENAL EFFICIENCY, WITH OBSERVATIONS ON THE "UREA COEFFICIENT."

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UNTIL recently the condition of the kidneys in patients suffering from renal disease was estimated chiefly on the results of an examination of the urine for protein and casts. While it was recognised that albuminuria might sometimes be present in otherwise healthy patients, the symptom was, on the whole, generally considered to be of great significance, indicating in the majority of cases a more or less grave lesion of the kidneys. When the albuminuria was accompanied by casts, clinical observers in general accepted the view that definite renal disease was present. Recent work, however, has shown that albuminuria is much more often met with than has hitherto been recognised, and it appears certain that many of our views concerning the importance of this condition must be subjected to a radical change.

In civil life it is difficult to ascertain the incidence of albuminuria in the population, but that it must be very much greater than has hitherto been supposed is evident from the results obtained in persons undergoing military service. In 50,000 apparently fit soldiers who had just completed their training, MacLean (1) found protein present in the urine of nearly 6 per cent. While many of these men showed only a small amount of protein, well-marked albuminuria was present in over 2 per cent., while in nearly 2 per cent. casts of various kinds were present as well. When it is considered that all these men had passed a medical examination before enlisting and that they represented the younger part of the population, it appears certain that an investigation on a similar scale of the average civil population would show an even greater incidence. All these men had just undergone several months of comparatively severe military training, yet they had not broken down or showed in any way that the presence of albuminuria rendered them less efficient than other men. That this large incidence of albuminuria was not connected with training was proved by an examination of 10,000 newly joined recruits. Here the incidence was, if anything, rather larger. This and other considerations compel us to modify our views on albuminuria; the condition may, of course, be an indication of a grave renal lesion, but albuminuria *per se* is not necessarily of any great importance, and is often not incompatible with the enjoyment of perfect health even in certain cases where it is known to have resulted from a previous attack of nephritis.

Further, as an index of renal efficiency the presence of albuminuria, even when associated with casts, is of little value in helping us to arrive at a conclusion in any given case. Generally speaking, the presence of very large amounts of protein together with granular and epithelial casts in the urine is, of course, an indication that the kidneys are more or less seriously involved, but even in such cases it is impossible to gauge to what extent these organs are still capable of performing their normal functions. This, after all, is the important point to decide, but this knowledge is not to be obtained by an examination of the urine for protein and casts. Other indications are, of course, available at the later stages of chronic renal disease, such as involvement of the cardio-vascular system, the low concentration of urea in the urine, the presence of œdema, etc., but in many cases of moderate severity no help can be obtained in this direction. In very severe cases the clinical condition alone is unfortunately often so grave as to give all the information required. On the other hand, it is not an uncommon occurrence in hospitals to have patients admitted who had been only very slightly indisposed before admission, but who rapidly become uræmic and die in convulsions a few days or a few weeks later. In such cases the clinical condition may remain good until a short time before the fatal issue. Usually, however, the gravity of the condition can be ascertained by an examination of the blood for non-protein nitrogen or urea. In severe cases the blood urea is found to be far in excess of the normal 20 to 40 mgrm. per 100 c.c. of blood. In acute cases also the estimation of blood-urea supplies the necessary information as to the progress of the patient. Unfortunately, however, the examination of the blood gives little or no help in the large number of cases in which the condition has not yet developed to an extreme degree, but in which the kidneys, though efficient enough to prevent an accumulation of urea in the blood, may yet be gravely involved. Such cases are very commonly met with at the present time in men who contracted war nephritis in the army; the presence of protein and casts in the urine does not help us, and in the great majority of such cases experience shows that the blood urea is normal.

THE NEW UREA CONCENTRATION TEST.

In endeavouring to ascertain the efficiency of the kidneys in such patients we have made use of the various dyes and other tests used so extensively in America, but on the whole, for one reason or another, we have not found them very satisfactory. We found, however, that when a large dose of urea was administered to a patient with defective kidneys the patient was *incapable of excreting urine with a high urea concentration.* Further, the degree of concentration appeared to be in direct relationship to the involvement of the kidneys. Reference has already been made to this test (2), but since that time we have been able to apply it in over 1200 persons suffering from the effects of war nephritis. The results have been most satisfactory, and the test has proved itself to be of very great practical value in assessing military patients for pensions. It is at present being extensively employed by the Ministry of Pensions for this purpose. The details of this test are as follows:

(1) The patient empties his bladder and immediately afterwards takes by mouth 15 grm. of urea dissolved in about 100 c.c. of water. The solution may be flavoured with a few drops of tincture of orange.

(2) One hour after taking the urea he passes water. This sample is kept for estimation of urea percentage.

(3) Two hours after taking the urea he again passes water. This sample is also kept for estimation of urea percentage.

The test is exceedingly easily carried out, the urea being estimated by the ordinary hypobromite solution. If the percentage of urea exceeds 2, the kidneys may be taken as fairly efficient; if below 2, the condition is unsatisfactory, and the lower the concentration the more serious the lesion. Cases with a concentration lower than 1 per cent. are rare, but many moderately severe cases are unable to concentrate to more than about 1·4 to 1·5 per cent.

The urea should be estimated by some form of apparatus which gives the volume of gas evolved. The ordinary apparatus giving percentage of urea directly is, in our experience, as a rule, not very accurately graduated. We use an ordinary 50 c.c. burette to act as a nitrometer, and take 4 c.c. of urine and 25 c.c. of hypobromite solution made up in the ordinary way. The open end of the burette is connected with a T-piece which communicates above with a glass tap or piece of rubber tubing fitted with a clip. By means of the side arm it is connected by rubber tubing with the bottle containing the urine and hypobromite solution. The lower end of the burette is attached by rubber tubing to a small bell-shaped vessel to hold the water displaced by the evolved nitrogen. By adjusting the level of the water in the nitrometer before and after an experiment in the ordinary way, satisfactory results can be obtained with the minimum of trouble.

The percentage of urea equivalent to the amount of gas evolved may be ascertained directly from the following table. This table was compiled by taking several specimens of urine and estimating the urea present by the very accurate urease method. Samples of the same urines were then analysed by the hypobromite method, and the amounts of nitrogen evolved compared with the amounts of urea known to be present in the particular samples. The results were then compared and an average taken.

Table showing the Percentage of Urea equivalent to c.c. of Nitrogen evolved.

(4 c.c. urine + 25 c.c. hypobromite solution used.)

Nitrogen c.c.	Urea per cent.	Nitrogen c.c.	Urea per cent.	Nitrogen c.c.	Urea per cent.	Nitrogen c.c.	Urea per cent.
8·0	0·5	22·4	1·40	36·8	2·30	51·2	3·20
8·8	0·55	23·2	1·45	37·6	2·35	52·0	3·25
9·6	0·60	24·0	1·50	38·4	2·40	52·8	3·30
10·4	0·65	24·8	1·55	39·2	2·45	53·6	3·35
11·2	0·72	25·6	1·60	40·0	2·50	54·4	3·40
12·0	0·75	26·4	1·65	40·8	2·55	55·2	3·45
12·8	0·80	27·2	1·70	41·6	2·60	56·0	3·50
13·6	0·85	28·0	1·75	42·4	2·65	56·8	3·55
14·4	0·90	28·8	1·80	43·2	2·70	57·6	3·60
15·2	0·95	29·6	1·85	44·0	2·75	58·4	3·65
16·0	1·00	30·4	1·90	44·8	2·80	59·2	3·70
16·8	1·05	31·2	1·95	45·6	2·85	60·0	3·75
17·6	1·10	32·0	2·00	46·4	2·90	60·8	3·80
18·4	1·15	32·8	2·05	47·2	2·95	61·6	3·85
19·2	1·20	33·6	2·10	48·0	3·00	62·4	3·90
20·0	1·25	34·4	2·15	48·8	3·05	63·2	3·95
20·8	1·30	35·2	2·20	49·6	3·10	64·0	4·00
21·6	1·35	36·0	2·25	50·4	3·15		

It occasionally happens that urea causes a marked diuresis in certain patients who have been imbibing large amounts of fluid immediately before taking the dose. The intake of excessive fluid may of course be prevented in hospital patients, but in the case of out-patients and others who are seen only for a short time it is sometimes impossible to avoid this complication. In such cases the very large amount of urine passed (often 400 to 600 c.c. in two hours) lowers the urea concentration even in kidneys that are capable of concentrating. Here the sample of urine for analysis should not be taken until the end of the third hour after the dose of urea is given. If it is not convenient to detain the patient beyond two hours, or if, as may very occasionally happen, the amount of urine passed in the third hour is also large, the condition may be put down to diuresis, especially in the absence of other symptoms of renal disease. The amount of urine passed during the second hour should not exceed 150 c.c. It is important to note that the passage of very considerable amounts of urine does not necessarily mean a low concentration of urea in the urine, for a quite healthy kidney generally succeeds in concentrating to 2 per cent. or over even after large amounts of fluid have been taken immediately beforehand. The mental condition also seems to play a part sometimes, as in a few cases we have noticed that very nervous patients tend to have a diuresis.

On the whole even in hospital out-patients there is very little trouble in the use of the test. The urea solution, though not pleasant to the taste, never appears to give rise to any untoward symptoms. In testing nearly 1500 cases only one patient rejected the urea by vomiting. The test is a simple one and very easily applied; it requires no special apparatus or facilities, and can be easily carried out in the hospital wards or in general practice.

ROUTINE TESTING OF RENAL CASES.

In testing for renal efficiency it is of course necessary to examine the cardio-vascular system as well as the kidneys. As a routine method we now make use of the following tests in the examination of very large numbers of cases which we are at present carrying out for the Ministry of Pensions. They are all easily applied, do not entail undue detention of the men, and appear to give all the information necessary for a just assessment of the patient's condition. In conjunction with the urea test we use the well-known diastatic test, but in many cases experience shows that this is really unnecessary. It is important to note that the diastatic activity of the urine is of much greater value when obtained from a sample of the twenty-four hours' output than in isolated samples passed at different times of the day. Of all these tests the new urea concentration test is the one which furnishes the most information, and our experience in nearly 1500 cases has led us to attach a very great deal of importance to it. Like every other test its results may in a very small percentage of cases be difficult to interpret, but its general reliability and ease of application should render it of great assistance in clinical medicine and in many surgical conditions as well.

Scheme for Examination of Renal Condition.

(1) Presence or absence of protein. Is amount very large, medium or small?

(2) Presence of casts or other abnormal elements, such as pus, blood, spermatozoa, etc. Nature and amount of casts present.

(3) Diastatic test (normal for healthy patient taken as 6 to 20 units).

(4) Urea concentration test.

(5) Presence or absence of œdema.

(6) Blood-pressure.

(7) Apex-beat position.

(8) Condition of arteries.

(9) General condition of patient.

This scheme is also used at St. Thomas's Hospital for the examination of both medical and surgical patients. In prostate and other operations on the urinary system it is most important to ascertain beforehand with certainty the condition of the kidneys, and we find that the above tests give us all the information required. In hospital practice we generally estimate the blood urea as well, especially in cases where the other tests indicate a more or less unsatisfactory renal condition.

The following results were obtained from patients: they illustrate cases where renal efficiency was good, fair, and bad. It is interesting to note that in each case fairly large amounts of protein together with large numbers of casts were present.

	Renal condition good.		Renal condition fair.		Renal condition bad.	
	Case 1.	Case 2.	Case 3.	Case 4.	Case 5.	Case 6.
Protein	Large amount	Large amount	Large amount	Very large amount	Fair amount	Medium amount
Casts	Hyaline and epithelial	Hyaline and granular	Hyaline, granular, and epithelial	Hyaline and epithelial	Hyaline, granular, and epithelial	Many epithelial
Diastatic test	20	10	6	4	5	1
Urea test	2·7	3·5	1·4	1·6	1·2	0·85
Œdema	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	Traces
Blood-pressure	126	120	138	128	230	190
Condition of arteries	Good	Good	Good	Good	Slight thickening	Slight thickening
General condition	Fairly good	Good	Fairly good	Fair	Anæmic	Fair (pale)
Blood urea (in mgrm. per 100 c.c. blood)	—	—	23	38	80	144

THE UREA COEFFICIENT.

In estimating the functional efficiency of the kidneys extensive use has been made in recent years, especially in America, of certain formulæ based on the relationship of the concentration of urea in the blood to the concentration and amount of urea in the urine. Widal and Javal (3), working on nitrogenous equilibrium in nephritis, showed that a rise in the blood-urea concentration could be produced by increasing the nitrogen of the diet, that such a rise was followed by a corresponding increase in the elimination of urea, and that ultimately nitrogenous equilibrium was re-established, though the blood-urea remained at a higher level than at the beginning of the experiment. The subject was further investigated by Ambard (4), who, from the laws which he had formulated for urea excretion, deduced the following constant, K, which he called the coefficient of urea excretion:

$$K = \frac{Ur}{\sqrt{D \times \frac{70}{W} \times \sqrt{\frac{C}{25}}}}$$

When Ur. = Grammes of urea per litre of blood.
D. = Grammes of urea excreted in twenty-four hours.
W. = Weight in kilos.
C. = Grammes of urea per litre of urine.
70 = Standard body-weight in kilos.
25 = Standard concentration of urea in grammes per litre of urine.

Ambard, working with this formula, found the value for K in normal human subjects to be 0·06 to 0·07, or according to F. C. MacLean (5), who worked with the more accurate method of urea estimation, 0·08. MacLean, eliminating the arbitrary standards for weight and concentration in the Ambard formula, employed the simpler expression $\frac{Ur}{\sqrt{\frac{D}{W} \times \sqrt{C}}}$, and obtained in a series of 107 observations of normal subjects figures varying from 0·20 to 0·36. The formula, therefore, gives fairly constant results in normal subjects, and the question arises how far variations from it are a regular feature of the function of the diseased kidney in which urea excretion is impaired.

With impairment of the urea-excreting function of the kidney two possibilities are present. In the first case the excretion by the diseased kidney may be able to keep pace with the formation of urea in the body, and the blood-urea will remain within normal limits even although excretion is not taking place along quite normal lines. In the second case, urea is not excreted as rapidly as it is formed and consequently accumulates in the blood, though equilibrium may be established at a higher level of blood-urea. In such cases blood examination reveals the excess of urea, and it only remains to determine whether the defective elimination is due to a primary kidney defect or whether it is secondary to some other condition, such as cardiac failure. With cases, however, in which there is no retention and in which the blood urea is within normal limits, the blood examination, as already indicated, gives us no information as to the extent of the impairment of kidney function, and it is in such cases that the Ambard constant should show a variation from the normal if it is to be of any practical value.

Taking the simple formula $\frac{Ur}{\sqrt{\frac{D}{W} \times \sqrt{C}}}$ certain possible defects are apparent. With a blood-urea within normal limits considerable variations in the factors D and C are necessary in order to produce any marked variation from the normal co-efficient. But if the blood-urea is to be kept down to a normal level, it is evident that the total amount of urea passed must be approximately normal and that the factor D/W will not vary. Secondly, little emphasis is laid on the concentration, for if we take extreme variations of C, as, for instance, 36, 25, 16 and 9 grm. per litre, and keeping the other factors constant at Ur 0·30 and D/W 0·35 work out the corresponding co-efficients, we get as results 0·20, 0·22, 0·25 and 0·30—all well within the normal limit. The formula as above expressed tends to diminish the importance of concentration of urea in the urine, and, by keeping the whole denominator at a fairly constant figure under conditions at all approximating the normal, to lay emphasis merely on the numerator, namely, the concentration of urea in the blood. That such a method could possibly be a delicate indicator of slight variations in kidney functions appears from theoretical considerations alone to be highly improbable, when we consider that it has long been recognised that a low urea concentration is characteristic of certain types of diseased kidney, even when the total amount of urea passed *per diem* approximates to the normal. Nothing is more striking in severe renal lesions than the failure of the kidney to concentrate to an appreciable extent even when the amount of urea in the blood is very high. For instance, in an extreme case the following values were obtained for urea in blood and urine:

Date.	Blood-urea per cent.	Urine-urea per cent.	Number of times urea of blood was concentrated by kidneys.
1 : 10 : '18	0·381	0·997	2·61
5 : 10 : '18	0·461	1·149	2·49
11 : 10 : '18	0·530	1·119	2·11
21 : 10 : '18	0·592	1·208	2·04

Since the normal concentration of urea in the urine is often more than 100 times that of the blood, it is obvious that the function of such a kidney is grossly defective even though a very considerable amount of urea, up to 20 grm. *per diem,* was being excreted.

It is the failure of concentration which is characteristic of the type of renal disease associated with azotæmia, and which, though it may be for a time compensated by an increase in the amount of urine passed, ultimately leads to an accumulation of nitrogenous products in the blood.

The urea concentration test already described furnishes a method by which the inability of the kidney to concentrate urea can be well brought out. Taking six cases of nephritis, in the first three of which retention of blood-urea was present, the following figures were obtained :

		Total urea passed in 2 hours.		Urea concentration in urine.
Group I	(1)	5·56 grm.		1·50 per cent.
	(2)	7·07 ,,		1·62 ,,
	(3)	5·94 ,,		1·26 ,,
	Average	6·19 ,,	Average	1·44 ,,
Group II	(4)	6·34 ,,		2·42 ,,
	(5)	5·42 ,,		2·79 ,,
	(6)	6·54 ,,		2·09 ,,
	Average	6·10 ,,	Average	2·42 ,,

It is obvious that while there is no appreciable difference in the *amount* of urea passed by the two groups under the conditions of the experiment, the first three cases in which urea retention was present show a very much lower urea *concentration* than the last three, in which no urea retention had at any time been noted. The more seriously damaged kidneys of Group I excreted the same quantity of urea as the less affected kidneys of Group II, but only by increasing the amount of water passed to an extent adequate to compensate for their defective power of concentration. While following a series of cases of war nephritis we were able to compare the results obtained by the urea test with the figures given by the formula $\frac{Ur}{\sqrt{\frac{D}{W} \times \sqrt{C}}}$ and to arrive at some conclusions as to the comparative value of the two tests as indices of the functional activity of the kidneys. The total quantity of urea passed *per diem*, the factor D, was calculated from the amount of urea passed during one hour, and in the course of this hour the patient was bled for the estimation of the blood-urea. This is the method usually followed by many of the advocates of this test, and for many reasons appears to be fairer than actually estimating the total amount of urea excreted during twenty-four hours. The diastatic index and the blood-pressure changes are given as confirmatory evidence of the course of the case. Where no value is given for the urea coefficient the patient was not in a condition to be weighed.

Case 1 :

Date.	Blood-urea per cent.	Urea coefficient.	Diastatic index.	Urea concentration test per cent.
25 : 9 : '15	0·2094	—	5	—
1 : 10 : '18	0·0710	—	6·6	—
8 : 10 : '18	0·0370	0·34	3·3	—
21 : 10 : '18	0·0475	0·46	5	—
8 : 11 : '18	0·0410	0·37	1·4	1·55
6 : 12 : '18	0·0292	0·33	2·2	1·55
27 : 1 : '19	0·0310	0·25	10	2·02

This was a severe case complicated by malaria, but improving rapidly. On discharge there were no cardio-vascular changes. Blood-pressure 120/73. Urine free from protein and containing only a few hyaline casts. The urea coefficient followed the fluctuations in the blood-urea content, and returned to normal limits before a normal concentration was reached with the urea test and while the diastatic index was still low. On discharge the urea concentration had just reached a normal figure, and the diastatic index was well within normal limits.

Case 2:

Date.	Blood-urea per cent.	Urea coefficient.	Diastatic index.	Urea concentration test per cent.
24 : 9 : '18	0·0715	0·68	1	—
2 : 10 : '18	0·1095	0·85	1	—
17 : 10 : '18	0·1015	0·88	1	—
4 : 11 : '18	0·0945	0·78	1	—
19 : 11 : '18	0·0582	0·46	1	1·27
7 : 12 : '18	0·0474	0·41	1	1·30
23 : 1 : '19	0·0360	0·36	2	1·37
29 : 2 : '19	—	—	1·5	1·30

Macroscopic blood was present in the urine until November 26th, 1918. On discharge the blood-pressure was 147/78, the radial artery was definitely thickened, the apex-beat was one quarter of an inch external to the nipple line, while the urine contained a small amount of protein with some granular and hyaline casts. The gradual fall in the blood-urea was associated with polyuria, the patient passing up to 100 oz. per day. The urea coefficient again closely followed the blood-urea, and though the last coefficient estimated was within normal limits, the raised blood-pressure, the cardiac hypertrophy and the persistent low diastatic index did not appear to indicate a very favourable prognosis. In keeping with this the urea test remained at a low figure.

Case 3:

Date.	Blood-urea per cent.	Urea coefficient.	Diastatic index.	Urea concentration test per cent.
31 : 8 : '18	0·0560	0·46	3·3	—
2 : 10 : '18	0·0373	0·51	4	—
14 : 10 : '18	0·0330	0·47	4	—
13 : 11 : '18	0·0286	0·35	4	1·43
16 : 12 : '18	0·0346	—	2·5	1·33
16 : 1 : '19	0·0340	0·40	2·2	1·83

On discharge there was still evidence of slight cardiac hypertrophy, the blood-pressure was 145/85, the radial artery being slightly thickened. The urine was smoky up to October 29th, 1918, and some epithelial casts were present. There was also a considerable amount of œdema.

Here the urea coefficient was with one exception slightly on the high side, in spite of the fact that the blood-urea was within normal limits. When, however, this normal coefficient was obtained, the urea concentration was very low, and the diastatic index was below normal. The urea coefficient in this case tended to be raised, owing to the comparatively small amount of urea passed and the very low concentration usually shown by this patient during the period of the test.

Case 4 :

Date.	Blood-urea per cent.	Urea coefficient.	Diastatic index.	Urea concentration test per cent.
13 : 9 : '18	0·0210	0·38	3·3	—
3 : 10 : '18	0·0678	0·94	2·2	—
25 : 10 : '18	0·0965	0·84	1	—
14 : 11 : '18	0·0745	0·71	1	—
4 : 12 : '18	0·0626	0·54	1	1·13
20 : 12 : '18	0·0468	0·45	1	1·12
24 : 1 : '19	0·0370	0·37	1	1·19

On discharge the apex-beat was external to the nipple line. The radial artery was definitely thickened and the blood-pressure 149 mm. of mercury. The urine gave a faint trace of protein and contained some granular casts. A definite polyuria was present. Phenolsulphonephthalein 18. The urea coefficient followed the blood-urea closely, and the improvement indicated by its fall in the later stages of the illness was not borne out by the diastatic index, the urea concentration test or the clinical findings.

Case 5 :

Date.	Blood-urea per cent.	Urea coefficient.	Diastatic index.	Urea concentration test per cent.
25 : 9 : '18	0·2643	—	6·6	—
1 : 10 : '18	0·1510	—	6·6	—
8 : 10 : '18	0·1180	—	10	—
14 : 10 : '18	0·0625	—	6·6	—
21 : 10 : '18	0·0570	0·43	6·6	—
4 : 11 : '18	0·0700	0·95	6·6	—
19 : 11 : '18	0·0488	0·43	10	1·46
10 : 12 : '18	0·0385	0·44	4	1·37
21 : 1 : '19	0·0325	0·27	1·1	1·41

On discharge the apex-beat was external to the nipple line, the blood-pressure 145/88, and the radial artery was moderately thickened. The urine contained a considerable amount of protein with a few epithelial and large numbers of hyaline and granular casts. In view of the severity of the attack, the low diastatic index and the clinical findings it did not appear that recovery was as complete as might be inferred from the urea coefficient. This view was in accord with the results of the urea concentration test.

Case 6:

Date.	Blood-urea per cent.	Urea coefficient.	Diastatic reaction.	Urea concentration test per cent.
? : 10 : '18	0·0334	0·47	2·2	—
28 : 10 : '18	0·0485	0·37	1·6	—
15 : 11 : '18	0·0445	0·48	1·2	1·55
4 : 12 : '18	0·0390	0·41	1	1·09
21 : 1 : '19	0·0335	0·37	1·4	1·25

On discharge there were no cardio-vascular changes; the blood-pressure was 122 mm. of mercury. The urine was faintly smoky, and contained a trace of albumen with a few hyaline, granular and epithelial casts. In this case the blood-urea indicated a tendency to retention, and an unfavourable opinion of the kidneys was confirmed by the results of the diastatic index and urea concentration test. The urea coefficient remained at the upper limits of the normal or at a definitely abnormal figure.

These cases represent a fair sample of the results of the various tests as seen in war nephritis and in ordinary civilian nephritis. As will be seen the urea coefficient tends to follow the blood-urea, and gives, in the majority of cases at any rate, no more information than can be obtained by an estimation of the urea in the blood. The results of Keith and Thomson (6), who employed the Ambard coefficient in war nephritis, are very similar, this coefficient usually rising or falling with the urea in the blood. When we compare, however, the normal coefficients obtained in such patients as 2, 4 and 5 on discharge with the other tests of functional efficiency and with the clinical findings, it becomes certain that recovery was not as complete as the urea coefficient would suggest. With a persistently low diastatic reaction and well-marked cardio-vascular changes it is difficult to believe that the kidney had completely recovered its function. In every case the urea concentration test bears out the clinical findings. In our opinion the urea coefficient is of very little, if any, value in cases in which the blood-urea is not raised. When the blood-urea concentration is higher than normal the urea coefficient rises with it, but it is only a waste of time and labour to utilise a complicated mathematical formula when the same information can be obtained from the urea blood concentration alone. In cases in which there is no tendency for the retention of urea the formula gives in the great majority of cases no useful information, and, indeed, tends to convey the impression that the patient's progress is much more rapid and his kidney efficiency much greater than is actually the case.

For facilities in carrying out this investigation we are indebted to the Medical Research Committee.

CONCLUSIONS.

(1) A new test of renal efficiency is described, depending upon the inability of the diseased kidney to excrete urea at a high concentration.

(2) This test is, in our experience, of more value in indicating deficiency of the kidney function than others now in use.

(3) A criticism of the value of Ambard's "coefficient of urea excretion" is offered.

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TECHNIQUE.

A SIMPLE METHOD OF RECORDING AUTOMATICALLY THE GAS PRODUCED BY BACTERIA IN CULTURE AND OF THE OXYGEN ABSORBED BY AËROBIC NON-GAS-FORMING BACTERIA.

(A Report to the Medical Research Committee.)

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Until the present time there has not been any method described by which the amount of gas produced by the growth of a bacterium can be automatically recorded. Methods have been used by which the gas given off was collected and measured at intervals and so curves of the gas-formation have been made.

The simplest method for collecting and measuring this gas is the one originally described by Beattie (*Brit. M. J.*, 1916, 1, 756). This consists merely in covering the surface of the implanted culture medium with melted vaseline. The vaseline is allowed to solidify and then the tube is incubated. The gas as it is formed collects under the vaseline plug and pushes it up the tube. The amount of gas can readily be estimated by measuring the distance to which the vaseline plug has been pushed up. This method one of us has used extensively in studying the effect of antiseptics on the growth and gas-formation of microbes in serum. It was felt, however, that it would be a great advantage if the amount of gas could be automatically recorded, thus avoiding the necessity for frequent readings and the possibility of an error in any of the readings. The apparatus described below is the one which we have devised for this purpose. It is simple and can readily be fitted up in any laboratory, as the only piece of apparatus necessary outside of the ordinary laboratory equipment is a slow-moving clockwork drum. The method is best described with reference to Fig. 1 and Fig. 2. Fig. 1 represents the whole apparatus and Fig. 2 represents diagrammatically the arrangement of the culture-tube.

A test-tube (A) containing a culture fluid (B) implanted with gas-forming microbes is taken and a layer of melted paraffin-vaseline mixture (C) is poured on the surface and allowed to solidify. A wooden disc (D) to which a thread is attached is then dropped on the surface and is covered to a depth of 0·5 cm. with the paraffin-vaseline mixture. When this solidifies the wooden disc is firmly embedded in a plug of the paraffin-vaseline mixture about 2 cm. thick. The thread (E) is then carried over a pulley-wheel (F) immediately above the test-tube and fixed to the end of a writing lever (G). This lever, which has its axis on a wheel (H), writes on a smoked clockwork drum (L). At the end of the lever to which the thread is attached there is hung a weight (K).

The whole of the apparatus is placed in the incubator and the gas as it is produced collects under the paraffin plug and pushes it up the tube. As the plug rises the weight attached to the lever will lower that end and so will raise the writing point which is recording on the moving drum. A continuous record of the gas-formation will thus be obtained.

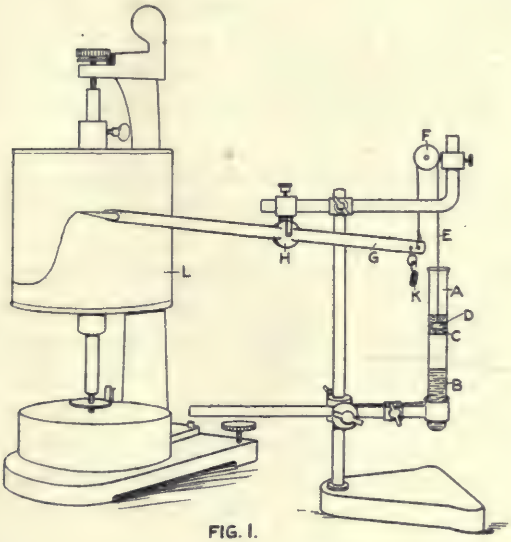
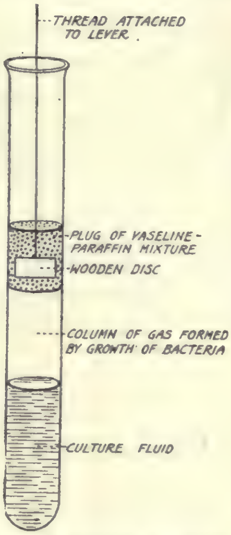


FIG. 1.


THREAD ATTACHED TO LEVER.
PLUG OF VASELINE-PARAFFIN MIXTURE
WOODEN DISC
COLUMN OF GAS FORMED BY GROWTH OF BACTERIA
CULTURE FLUID

TUBE ARRANGED TO RECORD GAS FORMATION.

FIG. 2.

There are certain details to which attention must be paid :

(1) The composition and preparation of the paraffin plug containing the wooden disc. Experiments were made using vaseline, but at incubator temperature this is very soft and the wooden disc is apt to be pulled right out of the vaseline.

Hard paraffin was found unsuitable as it did not slip sufficiently easily inside the test-tube.

The best mixture was found to be 4 parts of vaseline to 1 part of hard paraffin, and this mixture at incubator temperature slipped readily up and down the tube on the slightest force being applied, and at the same time it was firm enough that the wooden disc would not tear out, even if a comparatively heavy weight was applied.

Before pouring in the paraffin-vaseline mixture the walls of the tube above the culture fluid should be thoroughly dried by heating in the flame, otherwise the plug may not be air-tight.

(2) The weight (K) should be almost heavy enough to counteract the resistance of the paraffin plug to the pressure of the new-formed gas, so that the gas will be approximately at atmospheric pressure. We used weights of about 20 grm.

(3) The writing lever should be strong. There is no advantage in having a delicate lever. We found that corset steels or bicycle spokes made excellent levers when fitted with suitable writing points. The lever should be long, so that the distortion due to its circular motion is reduced to a minimum. Any desired magnification can be obtained by altering the position of the fulcrum (H). We generally used a two-fold magnification, but the most suitable arrangement would depend on the size of the drum and the size of the record desired.

(4) Any type of slow-moving clockwork drum would be suitable. The rate at which the drum should move varies with the type of investigation and the organism experimented with.

Thus, if it is desired to study the latent period before gas-production occurs a drum revolving in twelve or twenty-four hours would be suitable, whereas if a complete record of the gas-formation by a microbe like *B. sporogenes* is desired, a drum revolving once a week or slower would be more convenient. We found that a satisfactory arrangement was to have a 12-hour drum, and from the pin of this we could, by means of a waxed thread, turn another free-running drum. By varying the gearing (which is easily done by rubber bungs of varying sizes attached to the pole of the drum) the second drum could be made to revolve at the rate of from once a day to once a week.

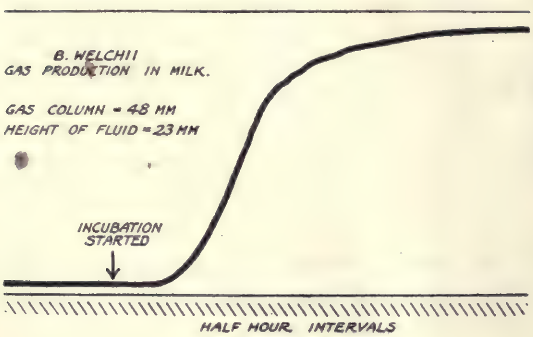


FIG. 3.

It is desirable to have a time marker recording half hours or hours, but if this is not available a mark can be made on the drum a certain number of hours after the commencement of the experiment and the other times calculated from this.

This communication is a purely technical one, so it is unnecessary to go into the results beyond giving the accompanying typical chart obtained by the method described.

The slight irregularities in the curve are due to slight variations in the temperature of our incubating room, as when there is a column of gas half-an-inch or more high the apparatus acts as an air thermometer. With a perfectly even temperature these irregularities would disappear.

The apparatus can easily be arranged to record the gas-formation in two tubes simultaneously by fixing up two parallel levers connected over two pulley-wheels, with two culture-tubes clamped side by side. By means of this double apparatus observations can be made on the amount of gas formed by two organisms in the same medium, or by the same organism in two different media, or many other comparisons can be made.

When the experiment is concluded the amount of CO_2 in the new-formed gas can easily be measured by pouring on to the surface of the paraffin plug an excess of 10 per cent. caustic soda solution and puncturing the plug so that this comes in contact with the gas. Some of the CO_2 is absorbed and by the caustic soda, and as it is absorbed a negative pressure is created which draws in more soda solution until the whole of the CO_2 is absorbed. The height of the column of gas is measured before and after and the reduction represents the amount of CO.

A similar method can be used automatically to record the oxygen absorption from the air by an aërobic bacterium (Fig. 4). A slight change must here be made as it is necessary to have the air-tight paraffin plug near the top of the tube at the beginning of the experiment. A support for the melted paraffin mixture is obtained by moulding some modelling wax into a thin sheet, and from this (with the top of a test-tube) a disc is cut which is carefully arranged to lie across the tube about 1 in. from the top. The paraffin mixture is poured on the top of this wax disc and then the wooden disc is embedded in it. The arrangement of the rest of the apparatus is as before.

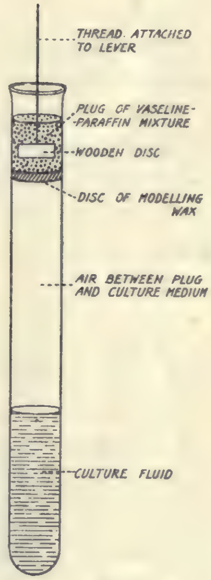


FIG. 4

Tube arranged to record oxygen absorption.

As growth occurs then some of the oxygen will be absorbed from the air of the tube and the plug will be sucked down the tube. This will cause the writing point of the lever to drop.

In conclusion we wish to thank Dr. Roaf for kindly supplying us with the recording apparatus necessary.

A LITMUS SOLUTION SUITABLE FOR BACTERIOLOGICAL PURPOSES.

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Perhaps litmus is more frequently used than any other substance as a test for the production of acid in media, chiefly in connection with the fermentation of sugars. For this purpose a reliable litmus solution of constant strength is of great value, and an essential if comparable results are desired. Prior to the war it was usual to buy the commercial solutions of litmus. When these were no longer to be obtained it was necessary to prepare them for oneself. None of the various formulæ tried, however, were found very satisfactory. The method given below has, in our hands, given every satisfaction after an extensive and prolonged trial. An important feature of the solution is that it keeps well.

PREPARATION OF THE LITMUS SOLUTION.

Weigh out 80 grm. of litmus granules, and measure out 300 c.c. of 40 per cent. industrial spirit.

To the 80 grm. of litmus in a mortar add 150 c.c. of the spirit. Grind well and then transfer to a flask and boil gently for one minute over gauze. Decant the fluid and add the remainder of the spirit to the granules; boil again for one minute. Decant and add to the first extract. Allow to settle overnight, or centrifuge. Do not filter. Decant into a large measure and make up to 300 c.c. with spirit. Add N/1 HCl drop by drop, shaking from time to time until the colour of the solution becomes purple (usually from 1 to 2 c.c. of the acid to each 100 c.c. of the extract).

The test for the correct reaction is to take a tube of tap-water and a tube of boiled distilled water and add a drop of the solution to each. The tap-water should show blue and the distilled water mauve. When making up media this extract is usually used 2½ per cent.

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THE VALUE AND MECHANISM OF THE COLLOIDAL GOLD TEST.

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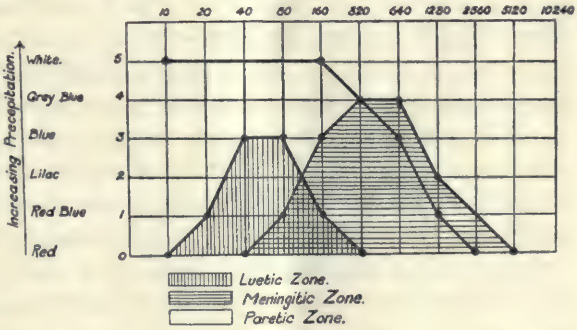
THE colloidal gold test of the spinal fluid was introduced by Lange in 1912, and has since been quite extensively employed by German and American workers in the diagnosis of mental and nervous diseases. In this country the test has not come into general use, although it is of considerable value in the routine examination of spinal fluids.

The application of a solution of colloidal gold to the examination of spinal fluid was suggested to Lange (1) by the work of Zsigmondy. The latter had shown that colloidal gold was sensitive to the presence of electrolytes, which caused coagulation and precipitation of the particles of gold, and that this reaction was prevented by the addition to the electrolyte of suitable amounts of various protein substances. These substances, which Zsigmondy called "protective colloids," showed varying degrees of protective action, and under certain standard conditions were specific for each substance. The amount of any protein which was necessary to protect 10 c.c. of gold colloid from the coagulating effect of 1 c.c. of 10 per cent. NaCl was the "gold number" of the protein. It occurred to Lange to examine syphilitic and normal sera by this method, and later to apply the reaction to spinal fluids, as the amount of globulin and albumen was known to vary in different pathological conditions of the central nervous system. Lange found, however, that certain spinal fluids, in addition to exhibiting protective effect on gold colloid, had also unexpected precipitating properties. The spinal fluids of cases of dementia paralytica in particular showed this precipitating property, and as a result of the examination of a series of cases Lange recommended the test for the diagnosis of this disease.

The conditions under which the Lange test is used are different from those which Zsigmondy used in obtaining his gold numbers. In the test of spinal fluid 0·4 per cent. NaCl solution is used, while in Zsigmondy's experiments 10 per cent. NaCl was employed. It will be shown in the course of this paper that the results obtained with strong and weak electrolytes are not connected, the mechanism being quite different in the two cases. This point is mentioned as it is evident from a review of the literature of the Lange test that the reactions have been confused.

The difficulty in applying the gold solution test to routine diagnostic work which all workers have encountered lies in the preparation of suitable samples of colloidal gold. Failures to prepare suitable colloidal solutions have occurred even in the hands of experienced workers, but with the knowledge which has now accumulated the difficulties are easily overcome. Absolutely standard colloidal solutions cannot be made, but practically all specimens can be made of service by simple adjustment of the reaction. It is essential to use pure distilled water, perfectly clean utensils, and pure reagents. The glass-ware should be made of hard glass, and must be thoroughly cleansed with nitro-hydrochloric acid, and repeated washing in distilled and in doubly distilled water. The water must be distilled at least twice over a glass condenser fitted with ordinary corks (not rubber), and should be used soon after distillation. Unsuccessful results are usually attributable to impure water or to imperfectly cleansed flasks. The principle of all the methods is that a weak solution of gold chloride in the presence of weak alkali is readily reduced to metallic gold by the action of certain chemical reducing agents. Any of the reducers in common use for photography are effective, but the best results for the purposes of the test are obtained with formaldehyde. The gold is thrown out in the form of extremely minute particles which, by their dispersion in the fluid, impart to it a deep red colour.

The two methods in common use are: (1) Lange's method, and (2) that of Miller, Brush, Hammers and Felton (2). Lange's method, orginally devised by Zsigmondy, is the simplest and probably the best. The reagents necessary are: (1) 1 per cent. gold chloride solution; (2) 2 per cent. potassium carbonate solution, and (3) 1 per cent. solution of pure formalin. 500 c.c. of doubly distilled water in a clean litre flask are heated to 60 c.c. over a large Bunsen burner, and 5 c.c. of the gold chloride and 5 c.c. of the carbonate solutions added. The temperature is raised rapidly to 90° C., and the flask removed from the flame. 5 c.c. of the formaldehyde solution are added quickly, drop by drop at first, in larger amounts as the reaction proceeds. During the addition of the formaldehyde the flask is very vigorously shaken. A faint pink colour appears which is quickly succeeded by a deep red. The colour is completely developed in less than two minutes. Occasionally the colour is purple or purplish red. These solutions should be discarded. A successful preparation should be a beautiful red fluid, clear by reflected and transmitted light. Fluids with a faint sheen may also be used, but distinctly opaque solutions are unsuitable. Gold solutions produced in this way show no tendency to settle out even after long periods of time, resist boiling and should be stored in the dark until used. The method of Miller and his co-workers is the same as the above except that 3·5 c.c. of 2 per cent. potassium carbonate are used, and that five drops of 1 per cent. oxalic acid solution are added when the temperature reaches 80° C. At 90° C. 2½ c.c. of 1 per cent. formaldehyde are added drop by drop with vigorous shaking. The colour slowly changes from pink through violet to a deep dark red. This method has been much used in America. According to Black, Rosenberg, and McBride (3), the oxalic acid is not only unnecessary but even inconvenient, in that it neutralises some of the potassium carbonate, which is necessary for the reducing action of the formaldehyde, and results in the production of acid solutions of colloidal gold. These authors have gone minutely into the chemistry of the reaction, and have determined the minimum and maximum amounts of alkali and formaldehyde which may be used. They recommend that the reaction of all samples should be tested, using 1 per cent. solution of alizarin red in 50 per cent. alcohol as indicator. This is done as follows: A double row of tubes is placed in a rack, and 1 c.c. of distilled water added to each tube. 1 c.c. of N/50 HCl is added to the first tube of one series, and 1 c.c. N/50 NaOH added to the first tube of the second series. With a pipette 1 c.c. of the contents of the first tube is transferred to the second tube, and this process of dilution from tube to tube is carried out down each series, the excess of fluid from the last tube being discarded. Two drops of 1 per cent. alizarin red in 50 per cent. alcohol are added to each tube, and 5 c.c. of colloidal gold. The indicator gives a yellow colour in presence of acid and purple with alkali. A red colour with a distinct tinge of brown is regarded as neutral. The amount of acid or alkali necessary to render neutral the whole sample of gold is readily calculated. By adjustment in this way many samples of colloidal gold can be made of service which otherwise would be of little use. It is probable that, if this procedure were adopted generally, results of more uniform character would be obtained. The final test of suitability of any sample of gold is that it should give a paretic reaction with the spinal fluid of a case of general paralysis and show no change with a normal fluid, and that 5 c.c. should be completely precipitated in one hour by 1·7 c.c. of 1 per cent. NaCl solution. In my experience the solutions produced by the original Lange method rarely require adjustment. Solutions of gold which are frankly acid will give reactions with normal fluids, while alkaline ones are not sensitive to paretic fluids.



In performing the test eleven small, dry, clean tubes are placed in a rack. To the first tube 0·9 c.c. of 0·4 per cent. salt solution is added; to the rest 0·5 c.c. A 1 in 10 dilution of spinal fluid is made in the first tube by adding 0·1 c.c. of spinal fluid. This is well mixed and 0·5 c.c. removed and added to the salt in the second tube. The fluid in the second tube is mixed, and 0·5 c.c. transferred to the third tube. This procedure is repeated down the series. In this way dilutions of 1 in 10, 1 in 20, 1 in 40, 1 in 80, etc., are made, the amount in each tube being 0·5 c.c. To each tube 2·5 c.c. of colloidal gold are added. A control tube containing 0·5 c.c. of a 0·4 per cent. salt may be added to the series to serve as a standard of colour. Various types of reaction may occur. These fall into three groups which have been designated the "paretic," "luetic" and "meningitic," or, as Felton and Maxy (4) recommend, zones I, II and III. These terms, though not rigidly descriptive, are so convenient that it is desirable to continue their use. It is customary to describe the changes of colour which occur in the presence of pathological spinal fluids by the numbers 5, 4, 3, 2, 1 and 0. The figure 5 represents a tube in which complete coagulation and precipitation of gold has occurred, leaving the supernatant fluid quite colourless; 4 indicates a pale grey-blue fluid, 3 a deep-blue colour, 2 a reddish violet, 1 a bluish red colour, and 0 indicates no change in colour. In the paretic type of reaction (zone I) complete precipitation of gold occurs in the tubes containing low dilutions of spinal fluid with a gradation of colour changes in the other tubes, *e.g.* 5 5 5 5 4 3 3 1 1 0 0. In the luetic reaction there is little or no change of colour in the first two tubes; the third, fourth and fifth are altered, the maximum change usually occurring in the fourth and fifth, but not exceeding the colour change represented by the figure 4, *e.g.* 0 0 2 4 3 1 1 0 0 0 0. In the meningitic type the colour changes appear in the tubes to the right of the series, the maximum change being in the seventh and eighth tubes and seldom exceeding 3. Complete precipitation of gold in the tubes in this zone has been observed very rarely in cases of meningitis. The paretic reactions are the most striking, owing to the flocculation and subsequent sedimentation of the particles of gold in a number of tubes and alterations in colour in the tubes to the right. In spite of the variation in the methods of preparing colloidal gold which different workers have used, the different zones have been described with considerable uniformity. The text-figure shows the three zones when the results are expressed in the form of a series of curves.

Since 1912 many papers have been published on the clinical value of the gold sol test, particularly in America. The experiences of various observers and the opinions formed regarding the practical value of the reaction may be summarised as follows:

Lange regarded the test as of great value in the detection of early cases of neurosyphilis, and more delicate than the Wassermann. Jäger and Goldstein (5) corroborated Lange's findings in a series of cases of dementia paralytica and cerebro-spinal syphilis. Kafka (6) considered that the gold test was more valuable than any other test of the spinal fluid. Sippy and Moody (7), from the examination of a large series of cases, were impressed with the sensitiveness of the reagent in cases of syphilis and were of opinion that the test surpassed the Wassermann as applied to spinal fluids in that it was qualitatively more exact, more sensitive, and required only a small amount of fluid. In their experience the gold reaction appeared very early and preceded all other signs in the course of dementia paralytica, and its value was enhanced by the frequency with which it showed early involvement of the meninges in secondary syphilis. Eicke (8) examined over 300 cases and obtained typical reactions in dementia paralytica, secondary syphilis and meningitis. He noted that the reaction in tabes was of the luetic type, and recorded a few cases of severe suppurative meningitis in which almost complete precipitation of gold occurred in the meningitic zone. Eskuchen (9) formed the opinion that the test possessed a degree of specificity and sensitiveness not obtained with any of Nonne's four reactions and proposed to call it the "fifth reaction." Solomon and Koefod (10) found that the syphilitic reactions ran parallel with the Wassermann tests of the spinal fluid. Solomon and Welles (11) found that the reaction in tabes was different from that of general paresis and characteristic of syphilis, but were unable to offer any conclusions regarding the value of the test in the differential diagnosis of general paresis and cerebro-spinal syphilis. Lee and Hinton (12) obtained gold reactions in 24 cases of tabes, of which 9 gave negative Wassermann reactions in both blood and spinal fluid. Of these 9 two gave no other spinal test positive. These patients all had a definite syphilitic history. In 8 cases of syphilis, without clinical evidence of involvement of the nervous system and with negative Wassermann reactions in the spinal fluid, 4 positive gold reactions were obtained. The blood Wassermann was also positive in these four cases. From their findings in a variety of cases they concluded that the gold reaction was nearly constant in syphilis of the nervous system and more delicate than other tests, and that the margin of error was small. No parallelism between the gold tests and the results of any other spinal fluid test was found. Miller and Levy (13) examined 210 spinal fluids from 171 cases, and found that all cases of paresis and tabes gave reactions in the paretic and luetic zones respectively. Luetic reactions were obtained in 13 out of 15 cases of latent syphilis in which there were no clinical symptoms indicating damage to the central nervous system, and in 80 per cent. of cases of congenital syphilis. They were convinced that the test was most valuable in differentiating the various syphilitic diseases of the nervous system, but were of opinion that it had no advantages over other laboratory tests in congenital, secondary, or tertiary syphilis. Further, the test did not distinguish between tubercular and suppurative meningitis. Flesch (14), as a result of his examinations, agreed with the findings of Miller and Levy. Grulee and Moody (15), on the other hand, disagreed with the conclusions of Miller and Levy in regard to the value of the gold test in congenital syphilis, and drew attention to the fact that Miller and Levy had obtained 100 per cent. of positive Wassermann reactions in this condition, a result which is not in agreement with the general experience of laboratory workers. Kaplan and McClelland (16) obtained characteristic curves in general paresis and in tabo-paresis, and luetic reactions in most cases of cerebro-spinal syphilis and tabes dorsalis. Weston, Darling and Newcomb (17), in a large series of mental cases, found the test as valuable as the Wassermann, but obtained "syphilitic reactions" in three cases which gave negative Wassermann reactions in the spinal fluid. In a later communication Weston (18) questions the relation of the gold test to syphilis, and gives details of three mental patients who, during a period of two years under repeated examinations, always gave paretic curves with the gold test and negative Wassermann reactions. In a footnote it is mentioned that one patient, tested while the article was in press, had developed a positive Wassermann reaction. Barnes and Ives (19) and also Swalm and Mann (20) applied the test in large numbers of mental diseases and found it of the greatest value, but did not consider syphilitic reactions were diagnostic of syphilis in the absence of other corroborative tests. Miller, Brush, Hammers and Felton, in a valuable paper, which has already been referred to in regard to the preparation of gold colloid, expressed the opinion that the gold test was more sensitive and specific in certain conditions than any other tests, and that the simplicity of the technique allowed of easy recognition of the sources of error. They described three cases of disseminated sclerosis giving paretic reactions.

Hammes (21), in a considerable number of syphilitic and other conditions, found no reactions with normal fluids and regarded the test as more delicate than any other. Lowrey (22) obtained no positive reactions in a series of non-syphilitic mental cases, but got 10 per cent. of atypical results. In a communication by Harvey (23) certain cases giving negative results with other tests and positive reactions with the gold test are recorded. Acute poliomyelitis has been examined by Felton and Maxy (24). They report the occurrence of luetic curves in the acute stage of the disease. A recent paper on the subject is that of Warwick and Nixon (25), who have examined 800 spinal fluids from 408 patients suffering from many different nervous and other conditions. These authors find that, while the various curves are not specific, they are of great diagnostic value in conjunction with other clinical and laboratory tests, and that they are of especial value in tabes and cerebrospinal syphilis. Positive gold tests were obtained more frequently in these conditions than were positive Wassermann reactions. Cases of multiple sclerosis are given which yielded either paretic or luetic types of reaction. Other cases of multiple sclerosis have been examined by De Crinis and Frank (26), Hammes, Miller and his associates, Kaplan and McClelland, Vogel (27), Solomon and Koefod, Flesch, Oetiker (28) and recently by Moore (29). Both paretic and luetic types of curves are recorded, and completely negative results in others. Twenty out of Moore's 28 cases gave paretic curves, with pleocytosis and altered globulin content, but all with negative Wassermann tests. Of the remaining 8 cases 3 gave luetic curves. Moore concludes that these 8 cases are probably not cases of multiple sclerosis and draws attention to the great difficulty in the clinical diagnosis of such conditions. In connection with the occurrence of paretic reactions in this disease it is interesting to speculate upon a possible relationship between these findings and the recent work of Bullock (30), who found that certain cases of multiple sclerosis yielded fluids which were toxic to animals, and the work of Kühn and Steiner (31), Simon (32), Siemerling (33) and Marinesco (34), who report the finding of spirochætes in experimental animals and in man. Another recent study of the colloidal gold reaction is that of Kyrle, Brandt and Mras (35), who have examined 720 cases of syphilis in all stages. They conclude that the gold reaction is specific in so far as it is more pronounced in syphilis than in any other conditions (with the exception of disseminated sclerosis), that it is much less likely to fail or yield doubtful results and that a positive reaction of any kind indicates a considerable change in the spinal fluid even if this is not shown by other reactions. A few other papers dealing with experiences of the reaction are given in local American journals and are quoted by Warwick and Nixon, but the papers which have been mentioned are sufficient to indicate the scope of the gold test in nervous, mental and syphilitic diseases.

As compared with the large number of cases which have been recorded above by certain workers, the series I have tested is small and confined to mental conditions. Certain observations bearing on the mechanism of the reaction are also presented, which are regarded as of interest in connection with a test which is of value in such a variety of conditions, and the nature of which is so little understood. These observations were for the most part made before the war, and the delay in publication is due to the interruption caused by military service. In the interval some other contributions have appeared which throw light upon the nature of the test and will be dealt with later.

The colloidal gold for these examinations was made by Lange's method, and after a little experience had been acquired no difficulty was encountered in making suitable preparations. Occasionally failures resulted, but these were easily overcome either by redistillation of the water or by greater attention to cleanliness of the glass-ware. Flasks of hard glass, preferably new, gave the best results. Samples of colloidal gold which did not react were rarely met with. These were titrated by the method of Miller, Brush, Hammers and Felton and after adjustment of the reaction were found to be quite suitable. The number of cases examined included 27 cases of general paralysis, 1 of juvenile general paralysis, 3 cases of suspect general paralysis, 1 of combined general paralysis and tabes dorsalis (tabo-paresis), and 58 miscellaneous cases comprising different mental conditions, chronic dementia, epilepsy, dementia præcox, delusional and other insanities. The spinal fluids were drawn off into a series of dry test-tubes and only the fractions which were free from blood to the naked eye were used for diagnostic work. In addition to the gold test the Wassermann reaction was carried out by the method of Browning, Cruickshank and Mackenzie (36). The amount of globulin was tested by the Ross-Jones procedure, also by Noguchi's butyric acid reaction, and in a few cases estimations were also made of the proportion of globulin to albumen. Cell counts of the fluids were not made in all cases and the results, therefore, are not embodied in the tables.

Table I gives the results which were obtained in the cases of general paralysis. They agree with those of other observers in that all cases gave paretic reactions. The Wassermann tests were also positive in all cases and various degrees of globulin increase were detected. No correspondence between the amounts of globulin in the fluids and the reactions with colloidal gold was found.

Table II shows a few cases which gave anomalous results with the gold test or in which there was discrepancy between clinical and laboratory diagnosis. Case 28 was a patient who had been diagnosed as a general paralytic on clinical symptoms. On admission the Wassermann reaction of the blood was positive, but lumbar puncture could not be carried out owing to the patient's excitable condition. Three months later spinal fluid was obtained and gave a somewhat doubtful positive Wassermann, showed slight increase of globulin, and moderate increase in the number of cells. The gold reaction was not tested at this time. The patient was treated with salvarsan intravenously and subsequently showed improvement in his mental condition. Three years later the spinal fluid was again examined. The Wassermann was negative and the anomalous gold reaction shown in the table was obtained, a reaction which approaches the luetic type but is not typical. As this patient at no time had any symptoms suggesting gumma or localised arterial change, and as he had suffered one congestive seizure in the course of his residence in hospital, the diagnosis of general paralysis was adhered to, but the possibility of cerebro-spinal syphilis could not be denied. Case 29 also presented difficulty in regard to diagnosis. The symptoms suggested general paralysis, but ocular signs were absent and no material change in symptoms occurred over a period of two years. The Wassermann and gold tests were performed on several occasions. The results were always of the kind shown in the table. Cases 30 and 31 are placed together as they were admitted about the same time, having very similar symptoms and both showing left-

TABLE I

Case No.	Wassermann Spinal Fluid.	Gold Reaction.	Globulin.
1	+	55543322100	+++
2	+	55543321000	+
3	+	55555543100	+
4	+	55544321000	++
5	+	55543321000	+++
6	+	54332210000	+++
7	?+	54332221000	+++
8	+	54432100000	+++
9	+	54333210000	+++
10	+	55543210000	+
11	+	55554321000	++
12	+	55532210000	+
13	+	55553210000	+++
14	+	55554321000	+++
15	+	55555421000	+++
16	+	55555421000	+++
17	+	44322111000	+++
18	+	55554321000	+++
19	+	55553221000	+++
20	+	55442210000	++
21	+	55554321000	+++
22	+	45454332100	+++
23	+	55555432100	++
24	+	5554332100	+
25	+	55555544210	++
26	+	4544332100	+++
27	+	5533221000	+++

TABLE II

Case No.	Wassermann Spinal Fluid	Colloidal Gold	Globulin
28	—	04332100000	+
29	+	13221000000	++
30	+	23333321000	++
31	—	11123321000	+
32	+	54433210000	+++
33	—	23221000000	++
34	—	10000000000	?+

sided paresis. Case 30 gave a positive Wassermann and a gold test which approached the luetic in type, and Case 31 a negative Wassermann and a meningitic type of reaction. The gold reaction in the latter was in all probability produced by a recent superficial hæmorrhage with meningeal reaction, as this type is mentioned by some observers as occurring in a few cases of cerebral hæmorrhage. The gold test in Case 30 suggested the diagnosis of cerebro-spinal syphilis rather than general paralysis. Case 32 was a juvenile general paralytic, all the reactions being the same as in the adult type of the disease. In Case 33 the diagnosis of tabo-paresis was made on admission on the basis of symptoms and the finding of a weakly positive Wassermann in the spinal fluid. The gold test was not done. Four years later there was little change in the patient's condition, the Wassermann reaction in the fluid was negative on two occasions, and the gold reactions were as shown in the table. Case 34 was a case with mental changes and nervous symptoms resembling those of multiple sclerosis.

The rest of the cases, comprising fifty-seven in all, and representative of various mental conditions, all gave negative Wassermann and gold reactions, and it is unnecessary to give the results in detail. Some of the fluids, particularly from cases of chronic dementia and epilepsy, showed distinct increase in the amount of globulin comparable to that found in some general paralytics, but caused no alteration in the gold colour. In the group of negative cases slight change in the colour of the gold in the first two tubes was commonly found. In three cases of chronic dementia reactions corresponding to mild meningitic types were found.

The spinal fluid from a young girl with acute meningitis was also tested. This fluid showed only slight increase in protein, contained practically no cells, but was turbid from the presence of pneumococci. The reaction with the colloidal gold was 0 0 0 0 1 1 1 0 0 0 0. The exceptional character of this fluid in respect to almost complete absence of any inflammatory exudate possibly explains the negative result with the gold test.

At the time the above tests were made little was known regarding the gold test beyond the various types of reaction in different diseases. Lange had expressed the opinion that the syphilitic and other reactions were caused by the appearance in the spinal fluid of mixtures of different proteins which were specific for these conditions. Zaloziecki (37), on the other hand, thought that the gold test was an immunity phenomenon. Jäger and Goldstein regarded the reactions as dependent on the electrical changes in the fluid and a purely physical change. Numerous other workers thought that specific proteins were responsible for the different zonal reactions and had obviously confused Zsigmondy's results with protective colloids and the precipitating action of spinal fluid. Matzkiewitsch (38) believed that the reacting substance in the fluids of general paretics was peptone, as he had found that the substance was dialysable and that gold colloid was sensitive to the action of small amounts of silk peptone. With the exception of this last work no attempt to analyse the various phenomena had been made. In 1916, however, Weston (39) published experiments which appeared to show that the Wassermann reacting substance and the gold reacting substance in paretic fluid were different in that the former was not dialysable while the latter dialysed out quite freely. He also showed that the reaction was probably not caused by the salts of the fluid or by the presence of amino acids. Miller and his associates also had found that the reacting substance was dialysable. In 1917, in a paper read before a meeting of the American Association of Physicans, Felton (40) stated that the reactions were dependent on the relative amounts of globulin and albumen, the globulin exercising a precipitating action and the albumen a protective action. Zonal reactions were due to the inter-relationship of the two substances, small amounts of albumen and globulin giving a reaction in the low dilutions and larger amounts a reaction in the high dilutions. Only a summary of the work has unfortunately been published and no details of experiments are given.

Early in the course of my examination of the gold reaction the association of positive Wassermann tests and paretic curves with colloidal gold suggested that a relationship existed between the two phenomena. As the reacting power of a syphilitic serum or spinal fluid has been shown to reside in the globulin fraction of the protein, paretic fluids were subjected to half saturation with ammonium sulphate in order to throw down the globulin. The precipitate was separated by the centrifuge, washed several times with half saturated ammonium sulphate in order to get rid of albumen, dissolved in salt solution and dialysed till free from salt. The precipitate of globulin was then dissolved in an amount of 0·85 per cent. NaCl solution equivalent to the original volume of spinal fluid. This fluid was tested with the gold test and with the Wassermann. It was found that the globulin had the precipitating power of the original spinal fluid and was even more active. For example, a fluid which gave the reaction 5 5 5 5 4 3 2 1 0 0 0 yielded a globulin solution which gave the reading 5 5 5 5 5 5 4 3 2 1 0. This experiment was repeated with many paretic fluids, always with the same result. The globulin also gave positive Wassermann reactions. Only slight diminution of the precipitating power followed prolonged dialysis of the globulin solutions. Dialysis of paretic fluids in thin celloidin sacs, which were extremely permeable to electrolytes and to peptone, did not appreciably diminish the precipitating action on colloidal gold, nor was any precipitating substance found in the dialysates even when large volumes were tested. Table III shows the results obtained in a typical experiment with a paretic fluid dialysed in a celloidin capsule for twenty-four and seventy-two hours. In each case sufficient 10 per cent. NaCl solution was added to restore the salinity of the dialysed fluid to the equivalent of 0·85 per cent. NaCl solution, as the globulin is only active when in solution.

TABLE III.

Paretic Fluid	5 5 5 5 5 4 3 2 1 0 0
,, ,, 24 hours dialysis	5 5 5 5 5 4 4 3 2 1 0
,, ,, 72 ,, ,,	5 5 4 3 3 2 1 1 0 0 0

In this experiment amounts of dialysate varying from 1·0 c.c. to 0·1 c.c. all gave no change with colloidal gold. These results are not in agreement with those of Matzkiewitsch, Weston and others, but these workers appear to have used for their dialysis the parchment thimbles which were recommended for use in Abderhalden's pregnancy test, many of which were extremely unreliable. Weston, in fact, found that his capsules permitted the passage of globulin into the dialysates, and these precipitated the gold sol.

The experiments were extended to the examination of spinal fluids which had given anomalous gold reactions. Globulin was separated from the spinal fluid of Case 28, dialysed and tested. A paretic reaction was obtained. Samples of spinal fluid from Case 29 were repeatedly examined in this way. The globulin invariably gave a paretic reaction. The experiments suggested that substances were present in the spinal fluids of these cases which interfered with the precipitating action of the globulin and caused anomalous results to appear. These fluids were therefore again precipitated with half saturated ammonium sulphate and both globulin and albumen fractions prepared and thoroughly dialysed. It was found that the addition of appropriate amounts of the albumen fraction to the globulin interfered with the precipitation of gold and caused anomalous results of the luetic type to appear. The albumen fraction in fact acted as a protective colloid. Other albuminous fluids, which by themselves had no power to precipitate gold colloid, also possessed protective powers. Comparatively small amounts of human or animal serum albumen exerted protective action, and it was possible by adjustment of the amount to render completely negative the action of the globulin on colloidal gold. In many of these experiments it was very difficult to give a reading of certain tubes in the usual numbers as there was frequently distinct turbidity only with no change in colour. The protective effect of albumen was also demonstrated by adding albumen to paretic spinal fluids, the albumen being added to the usual amount of spinal fluid in the first tube only and the appropriate dilutions then made with 0·4 per cent. NaCl solution. For example, the addition of 0·1 c.c. of 1 in 5 dilution of human serum albumen converted a marked paretic reaction into a meningitic one with the reading 0 0 0 0 1 1 3 3 2 1 0.

Cerebro-spinal fluids which gave negative results with the gold test were now examined. It was found that the globulin from these fluids did not precipitate colloidal gold. Even when large volumes of negative fluids were precipitated with ammonium sulphate and the globulin taken up in small volume of salt solution so that the concentration of globulin was from 50 to

100 times that of the original fluid there was only slight precipitation of gold colloid when the concentrated globulin was tested in the usual way. These experiments suggested that the reactions of paretic fluids were not due solely to the increased content of globulin, but to an alteration in the physical or chemical character of the globulin, and that the different zone reactions were dependent on the relative amounts of globulin and albumen, as Felton has suggested. As it has been shown by several workers that the relative amounts of albumen and globulin in the spinal fluids from cases of syphilitic and other diseases of the nervous system vary within wide limits, the possibility of different types of reaction with colloidal gold resulting from such variations is considerable. The way in which the protective action of albumen is exerted is difficult to understand. The protective effect is not shown on the tubes with greatest dilution of spinal fluid, but most markedly on the tubes of low dilution—that is to say, the zone reactions move from left to right of the series, not, as might be expected, from right to left. In fact, by gradation of the amount of albumen, paretic reactions may be converted into luetic, meningitic or negative reactions. Zone effects may also be demonstrated if varying amounts of paretic fluids are added directly to colloidal gold without the use of salt as diluting fluid. Table IV shows the results obtained in this way with various spinal fluids, two of which are paretic, one from Case 29 and the other a fluid giving a negative reaction.

Table IV.

(In each tube 2·5 c.c. of gold colloid.)

	Amounts of cerebro-spinal fluid in c.c.					
	0·05	0·1	0·25	0·5	1·0	2·0
Case 25	5	5	0	0	1	1
„ 20	2	2	5	2	3	2
„ 29	0	0	0	1	2	4
„ 59	0	0	0	0	1	1

It will be seen that complete precipitation of 2·5 c.c. of gold colloid occurs with 0·05 c.c. of the spinal fluid of Case 25, whereas no change is produced by the addition of 0·5 c.c. of the same fluid to the same amount of gold. If the amount of colloidal gold in the tube containing 0·5 c.c. is later increased to ten times (25 c.c.) complete precipitation of this large volume results. That is to say, there is a definite relationship between the amount of colloidal gold and the amount of cerebro-spinal fluid which will cause complete precipitation.

Spinal fluids which are active in precipitating colloidal gold under the conditions of Lange's test also possess protective power under other conditions, and this applies both to the globulin and to the albumen fractions. To demonstrate this it is necessary to use an amount of strong electrolyte which by itself precipitates colloidal gold. If to a series of tubes, each containing 0·15 c.c. of 10 per cent. NaCl solution, graded amounts of a paretic fluid are added it will be found on adding 2·5 c.c. of colloidal gold to each tube that the tubes with large amounts of spinal fluid show no precipitation, while the tubes with insufficient fluid show the colour change and precipitation produced by this amount of salt. Differences in the protein content of spinal fluids can be elicited in this way, those with most protein showing the greatest protective effect. Table V shows the results of such a test.

TABLE V.

(In each tube 0·15 c.c. of 10 per cent. NaCl and 2·5 c.c. colloid.)

	Amount of cerebro-spinal fluid in c.c.								
	0·06	0·05	0·045	0·04	0·035	0·03	0·02	0·015	0·01
Case 12	0	0	0	0	1	2	4	5	5
Case 60	1	2	3	3	4	4	5	5	5

By varying the strength of electrolyte it is possible to show in one series both the precipitating action and the protective one. The protective effect of a cerebro-spinal fluid or serum albumen is not destroyed by boiling, but the precipitating power is completely abolished. If the coagulated protein is removed by filtration or by centrifuging, the protective effect of the fluid is not lessened and may even be increased.

As serum albumen acts protectively it would be expected that the presence of blood in spinal fluid would interfere with the gold test. If the fluid is a paretic one the presence of considerable amounts of blood does not mask the reaction. Slight discolouration of the tubes in the luetic and meningitic zones occurs with negative fluids and no reaction of this type is of value in diagnosis if blood is present in the fluid. Fresh human serum precipitates colloidal gold in high dilutions. Half a cubic centimetre of 1 in 300,000 dilution may produce complete coagulation and precipitation of 2·5 c.c. of gold colloid. A dilution of 1 in 100 serum, used in place of spinal fluid in the ordinary test, will produce a typical paretic reaction. Zone phenomena are frequently encountered in the use of serum, but it is common to find complete sedimentation produced by the use of 0·5 c.c. of all dilutions of serum down to 1 in 300,000. A number of human sera have been examined in this way. Slight variation in activity exists in the sera of different persons, but there is no quantitative difference between syphilitic and normal sera in this respect. As in the case of spinal fluid the reacting substance resides in the globulin. With the globulins zone phenomena are seldom encountered. The albumen fraction of serum has strong protective powers, but has no precipitating property. A 1 in 1000 dilution of serum used in the ordinary test causes the production of a typical meningitic reaction, and it seems probable that the reactions of this type in acute and sub-acute meningitis are caused by leakage of serum proteins through the inflamed meninges. It would be interesting to separate the globulins from meningitic spinal fluids and compare the activity with those of normal and paretic fluids and to determine the degree of protective action exerted by the albumen fractions. Human serum quickly loses its activity when kept at room temperature under sterile conditions and within a week may have no precipitating action. Nevertheless, the globulins prepared from such old sera are as active as the fresh serum or fresh serum globulin. Human serum differs from most animal sera in its effect on colloidal gold. Sera from the rabbit, guinea-pig, sheep, horse, ox, fowl, mouse and monkey, have all been tested and little or no alteration of gold colloid is produced. The serum of the rat, however, precipitates gold, though it is less active than that of the human subject. This reaction of different sera is at present under examination.

The change which results when paretic fluids or human sera are added to colloidal gold is different from that which is produced by electrolytes, peptone, or other chemical reagents. With the latter the change of colour to deep blue is immediate and precipitation of gold is rapid. In the case of paretic fluid and serum there is little change of colour, the fluid becomes turbid, the particles of gold settle out slowly and the deposit is reddish-violet in colour, not blue, as is the case with electrolytes. The particles of gold in a solution of colloidal gold are negatively charged and will be flocculated by positive colloids and by the cations of electrolytes. The coagulating power of an electrolyte for a negative colloid varies with the valency of the cation and the higher the valency of the cation the smaller is the amount of electrolyte required to bring about coagulation and precipitation. In the performance of the gold test the amount of spinal fluid used is small and precipitation occurs in such a high dilution that it is extremely improbable that the precipitating substance is an electrolyte. Only an electrolyte of high valency could produce the effect, and it would require to be attached to the globulin fraction in such a way that it is not freed by dialysis. It has been demonstrated that, when flocculation is caused by electrolytes, the particles of colloid unite with the ion of opposite electric charge and that the sediment is composed of both substances. An analysis of the sediment which results when a paretic fluid is mixed with gold colloid would determine the nature of the substance causing precipitation. In a series of experiments on this point I have found that, by adding an amount of spinal fluid directly to gold colloid so that complete precipitation just results, the clear supernatant fluid shows complete absence of globulin, whereas control experiments, using the same amount of spinal fluid in a volume of salt solution equal to that of the gold colloid, show the presence of globulin. This experiment supports the other findings that the substance concerned in the precipitation of gold is the globulin fraction of the fluid. The reaction of paretic fluids is removed by shaking the spinal fluid with such substances as kaolin and fuller's earth. This is due to the removal of protein by adsorption. The substance which reacts with colloidal gold resists prolonged exposures to temperatures which fall short of causing coagulation of the protein.

Paretic fluids which are kept under sterile conditions do not lose the precipitating property, and in this respect differ from serum. One author found that a spinal fluid was as active after a year as in the beginning. This is of great advantage, as the same fluid may be used repeatedly in order to test different samples of gold colloid. Spinal fluids obtained post-mortem give the same reactions as ante-mortem fluids.

Samples of colloidal gold made by other methods—for example, by effecting reduction with dilute tannic acid or by a solution of phosphorus in ether—have been tested and found to be unsuitable for the test of spinal fluid. Such colloids are precipitated by paretic and by normal fluids. After prolonged dialysis against pure distilled water these colloids give the same reactions as gold colloid prepared by the use of formaldehyde.

The addition of small amounts of acid to paretic fluids increases the zone of precipitation, while the addition of alkali decreases it. Acid solutions of gold tend to show precipitation with negative fluids and distinctly alkaline ones are not sensitive to paretic fluids. The importance of adjusting the reaction of each sample of gold for use in the test is evident.

SUMMARY AND DISCUSSION.

The results of other workers regarding the value of the gold test in the diagnosis of cases of general paralysis have been confirmed. No parallelism between the gold reactions and those of other tests has been found. The substance in the spinal fluid of general paralytics which causes precipitation of colloidal gold is not dialysable, and resides in the globulin fraction of the protein, and in this respect resembles the Wassermann reacting substance. The substance is not altered by prolonged heating at temperatures short of those which cause coagulation of the protein. No evidence has been obtained which supports the view that the reaction depends on the presence of peptone. The various types of reaction can be simulated by mixtures of globulin and albumen, the globulin acting as a precipitating agent and the albumen as an inhibitory or protective one. Luetic reactions are due to an amount of albumen sufficient to obscure partially the precipitating effect of the globulin. As the globulins obtained from negative spinal fluids, even when used in concentrated form, are almost inactive, the precipitating action of paretic fluids cannot be ascribed solely to the increase in globulin, but is probably dependent on a specific alteration of the physical state of the globulin, which is associated with a positive electric charge. This alteration in the globulin cannot be regarded as specific for syphilis, since it occurs, according to the work of others, in multiple sclerosis, but it is interesting that the change is so constant in one spirochætal disease and common in another which is now suspected to be due also to a spirochæte. The test might serve to separate cases of true multiple sclerosis from those diseases which clinically simulate it very closely. Human serum globulin also possesses the characters of a positive colloid, but the paretic and luetic reactions of spinal fluids cannot be attributed merely to the passage of serum globulin into the spinal fluid. The meningitic reactions, on the other hand, may be due to the leakage of plasma through meninges damaged by inflammatory change, and are probably of quite a different order to those of syphilis and multiple sclerosis. In reviewing the results obtained by other workers it is evident that for the most part difference of opinion exists in regard to the interpretation which should be put upon reactions of the meningitic type. The fact that this type occurs in such a variety of conditions supports the view that it is dependent on meningeal irritation, which may be caused by tumours, hæmorrhage or inflammatory conditions. The impression has been formed that for the present only reactions of the paretic and luetic types should be regarded as of real diagnostic value, and that the meningitic reactions, while being of some value in conjunction with other findings, should for the present be held *sub judice*.

CONCLUSIONS.

1. In general paralysis the results of the colloidal gold test coincide with those given by the Wassermann reaction. There is a considerable degree of probability that in those cases in which the colloidal gold is positive with a negative Wassermann the gold test is the more delicate indication.
2. No parallelism between this test and the other spinal fluid tests has been found.
3. The reacting substance in paretic fluids is not dialysable and resides in the globulin.
4. The reaction in syphilitic diseases of the central nervous system depends on a physical change in the globulin associated with increased positive electric charge.
5. The zone reactions depend on the relative amounts of globulin and albumen, the globulin having precipitating action and the albumen a protective one.

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THE EXPERIMENTAL TRANSMISSION OF *ENCEPHALITIS LETHARGICA* TO A MONKEY.*

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Soon after v. Economo (1) had drawn attention to the prevalence of *encephalitis lethargica* in Vienna in 1917, v. Wiesner (2) with material obtained from v. Economo's cases, produced a hæmorrhagic encephalitis in a monkey.

The monkey was inoculated subdurally with 0·2 c.c. of a brain emulsion from a fatal case of encephalitis. The effects produced were briefly as follows: The animal recovered completely from the operation, but a few hours later it was noticed to have become very quiet and to sit on the floor of the cage. Next morning it remained in a corner of its cage with its eyes usually shut, and it appeared to be "somnolent." It responded, however, to calls and would still eat. But left alone it shut its eyes and slumbered uninterruptedly. The picture was exactly that of a person struggling to prevent himself falling asleep. There was slight paresis of the right hind foot. Later in the day the sleepiness and the feebleness had markedly increased. The walk had now become ataxic. Death occurred next day, forty-six hours after the inoculation. For some hours previously the animal had been completely apathetic, had lain on the floor of the cage with closed eyes, and had no longer responded to stimuli. At the necropsy, sections of the brain showed a picture typical of acute hæmorrhagic encephalitis affecting the whole cortex and medulla. There were also considerable œdema and hyperæmia. A diplococcus was isolated from the brain. No detailed account is given of the microscopical changes produced. The writer considered the macroscopic changes to be sufficiently diagnostic. Filtered emulsions of the above monkey's brain and emulsions of pure cultures of the pleomorphic coccus failed to produce any fatal illness or symptoms of note in other monkeys.

More recently Strauss, Hirshfeld and Loewe (3) claimed to have successfully inoculated monkeys with brain emulsions and nasal washings from cases of encephalitis.

In their first experiment a *Macacus rhesus* monkey was inoculated subdurally with a saline emulsion of an encephalitis brain. Next day the animal was found to be ill and the spinal fluid contained ninety mononuclear cells per c.mm. Two days later a necropsy showed

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[*] A note on this experiment appeared in the Forty-eighth Annual Report of the Local Government Board, 1918–1919. Medical Supplement. London, 1919, p. 76.

multiple hæmorrhages in the brain, a moderate meningitis and a perivascular infiltration with mononuclear leucocytes. Monkey No. 2 was inoculated with a brain emulsion from this monkey No. 1. The animal showed six days later a hemi-paresis on the side opposite the inoculation. From the appearances seen at the necropsy the authors suggest that the lesion was probably traumatic. Monkey No. 3 was also inoculated with material from the first monkey but the result was quite negative. Monkey No. 4 was inoculated with the filtered washings of the naso-pharynx from a case of encephalitis. The inoculation was made subdurally and intra-peritoneally. Eight days later there was paresis of both hind legs, and the animal was apathetic. The animal recovered completely. Monkey No. 5 was inoculated as above with naso-pharyngeal washings. The day after inoculation there were observed a right hemi-paresis and a paresis of the external rectus of the left eye. Three days later the animal developed a right-sided Jacksonian epilepsy which ended in stupor. The condition got worse, and the monkey was killed on the sixth day. The necropsy showed a condition similar to that of monkey No. 1, and a large hæmorrhage in the pons. Monkey No. 6 was used as a control, and monkey No. 7 was inoculated with material from monkey No. 5. In monkey No. 7 there was persistent paresis of the right arm and leg for two to five days after the inoculation. The necropsy showed a condition very similar to that of monkey No. 1, and in addition a large hæmorrhage with necrosis at the site of the peritoneal inoculation.

In a later communication (4) these writers state that they have transferred the disease to rabbits. The short incubation period in their monkeys, and the type of lesion produced, suggest that these authors were dealing with some virus other than that of *encephalitis lethargica*. Thus, in our experience, gross hæmorrhages have not occurred in cases of human *encephalitis lethargica*. Their experimental lesions resemble those obtained by v. Wiesner, and more recently in rabbits by Sherwood and Downs, with a strain of pleomorphic streptococci. The fact that Strauss, Hirshfeld and Loewe failed to obtain any bacterial growths does not entirely exclude infection by pleomorphic streptococci, because v. Wiesner pointed out that it was only by the employment of special methods that he succeeded in growing his cocci from infected material. Until the lesions produced and the clinical histories of the cases furnishing the material are given in greater detail, the claims of the above authors to have produced *encephalitis lethargica* in animals must be accepted with reserve.

In a previous communication (6) dealing with a number of bacteriological and experimental investigations on *encephalitis lethargica* an account was given of attempts to transmit the disease to monkeys. Material from eight fatal cases, which had shown what are generally considered to be characteristic features of the disease, both from the clinical and histological aspect, was inoculated intra-cerebrally and intra-peritoneally into *Macacus rhesus* monkeys and rabbits. With one exception no definite paralysis was observed in the monkeys, even after an interval which might be considered as a very long incubation period. One or two of the monkeys, however, some ten days after the inoculation showed some degree of excitement and trembling such as are frequently observed before the onset of experimental poliomyelitis. These signs passed off in a day or two and the animals regained their usual vigour.

The one exception was a monkey which developed a flaccid paralysis of the hind legs eighty-four days after inoculation. The paralysis gradually became complete and appeared to be similar to that observed in experimental poliomyelitis. The necropsy showed, however, that the paralysis was due to pressure from a tuberculous granuloma of the lumbar vertebræ.

Since the above attempts several more have been made, but without result until the present recorded instance.

The successful inoculation was obtained with material derived from a case which occurred in a small epidemic in a Home for girls at Derby. For the material and the notes on the epidemic we are indebted to Dr. A. S. MacNalty, who furnished us with a full account of his investigations, of which the following is an extract.

HISTORY OF EPIDEMIC AT DERBY.

At the time of the outbreak there were resident in the Home eighteen girls and a staff of three women. All the girls were said to have been in robust health before the outbreak; none had been attacked by influenza during the epidemic of that year. The majority of the girls were engaged in laundry work, the remainder in housework.

The outbreak commenced on August 14th, 1919. When Dr. MacNalty visited the Home on August 20th all three of the female staff and eight of the girls had been affected. The illness had commenced on the following dates: August 14th, two cases; August 15th, two cases; August 16th, four cases; August 17th, two cases; August 19th, one case. A twelfth case was reported after Dr. MacNalty's visit.

In analysing the clinical features of the outbreak Dr. MacNalty divided the cases into two groups.

Group I.—Abortive Cases.

Of these there were three. The patients were the members of the staff. Their ages were: 55, 34 and 30 years. The symptoms were slight, comprising nausea or slight vomiting and diarrhœa, "fainting turns" and stiffness in the muscles of the neck. One patient had slight bilateral ptosis, most marked in the left eyelid. With the exception of the ptosis, which was still present in this case on August 20th, a slight degree of prostration was the only sequel of a single day's illness. One of the patients remained in bed on the day of illness. The other two continued at their work.

Group II.—Manifest Cases.

Of these there were, up to August 20th, eight cases. The patients were all girls domiciled in the Home. Their ages were: 15, 16, 17, 17, 17, 17, 18 and 22 years. The symptoms and signs were as follows:

Temperature.—The temperatures were not recorded accurately or systematically. An initial, slight rise of temperature seems to have been present, ranging from 99° F. to 101° F.

The symptoms at onset were: Soreness or stiffness of the throat, nausea, in two or three cases actual vomiting, inability to see properly, drowsiness and vertigo, and, in two cases, nose-bleeding.

Ptosis and ophthalmoplegia.—All the cases had bilateral ptosis, and widely dilated pupils with sluggish reaction to light and accommodation. The ptosis was typically progressive and unequal; in the earlier cases it was extreme, in the later it was less.

Other paralyses.—In three cases the palatal reflex was lost, and there was a paralysis of the larynx, which caused complete or almost complete aphonia. The sphincters were not involved. There was neither retention nor incontinence of urine.

Peripheral reflexes.—These were exaggerated. Kernig's sign was present in nearly every case.

Rigidity and spasticity of the lower limbs was conspicuous in one case.

Tremors and irregular jerking movements of the limbs were displayed by two cases.

Voice.—The majority of the patients had a monotonous, nasal voice with a tendency to slurring speech.

Lethargy.—Seven out of the eight cases were, or had been, definitely lethargic. On August 20th, in three cases the patients were in a semicomatose condition and the stupor had greatly intensified; in two cases the patients were still very lethargic; in one case the lethargy was diminishing; in one case lethargy had deepened into coma, and the patient had died.

In the eighth case the patient had been drowsy, but not definitely lethargic. This case was, in general, mild in type. The pupils were only slightly dilated, and loss of vision had ceased to trouble the patient.

In all the cases examined on August 20th, the patient could be roused out of her lethargy and would answer questions; if left alone, she soon relapsed into stupor. Three of the patients displayed emotional symptoms when roused.

Facies.—All the cases exhibited a facies devoid of expression. One patient, who was seriously ill, had a bright pink flush on the malar bones, and showed champing movements of the jaws.

Fatal Cases.

Of these eight cases in which the illness commenced between August 14th and August 19th, four terminated in death. The age, date of onset of illness and date of death were as follows:

S. A—, aged 17 years. Onset, August 14th ; death, August 22nd, 1919.
G. P—, aged 18 years. Onset, August 15th ; death, August 25th.
V. S—, aged 17 years. Onset, Angust 16th ; death, August 25th.
M. C—, aged 17 years. Onset, August 16th ; death, August 19th.

No cases of *encephalitis lethargica* or of infantile paralysis had been recently notified in the district, nor had the Medical Officer of Health heard of any cases of obscure illness.

The above extract from Dr. MacNalty's full investigation of the outbreak should suffice to demonstrate the accuracy of his conclusion that "*from clinical examination the cases appear to be identical with cases of encephalitis lethargica.*"

The brains from the patients M. C— and S. A— were sent whole in glycerine to the London Hospital. Unfortunately both Dr. McIntosh and Dr. Turnbull were absent on holiday at the time. The brain from M. C— was delivered to the Pathological Institute. It had been in glycerine for twenty-four hours. Dr. W. W. Woods, having immediately removed and placed in 4 per cent. formaldehyde portions for microscopic section, sent the brain to the Bacteriological Department, but when subsequently examined it was considered to be too far decomposed for the purpose of inoculation. A complete history was obtained by Dr. MacNalty of the illness of the patient from whom this brain came. This history and the result of the histological examination of the brain will be given in detail in a subsequent paper. The signs of illness were disturbance of vision, a dry feeling in the throat and mouth, inability to swallow, and increasing lethargy, which gave place to restlessness and emotion when the patient was roused. The illness was more rapidly fatal than in any of the other cases, death occurring within a few hours more than three days after the onset. No active inflammatory reaction was present in microscpic sections of the right frontal, parietal and occipital lobes, the anterior and posterior segments of the mid-brain, the upper extremity of the pons and the upper extremity of the cervical cord.

TECHNIQUE OF TRANSFERENCE OF THE VIRUS TO ANIMALS.

The brain (Case S. A—) from which the material used for the inoculations was derived had been placed in 33 per cent. glycerine and despatched to the London Hospital. But, as already stated, owing to its arrival during the holidays the complete brain had been allowed to remain in the original glycerine for about fourteen days. The brain was found to be very soft. Pieces of the cervical cord, pons and basal nuclei were removed and placed in fresh glycerine for inoculation into animals. Portions of the cervical cord and pons were at the same time placed in formaldehyde solution for histological examination, but the mid-brain was considered to be much too soft for this purpose.

In the *microscopic sections* of the pons and upper extremity of the cervical cord there were no hæmorrhages and no trace of active inflammatory reaction.

For the experiments two monkeys were used : a *Macacus rhesus* and a *Cercopithecus patas.* Both monkeys were inoculated intra-cerebrally and intra-peritoneally. Pieces of the cervical cord, pons and basal nuclei, each about the size of a large pea, were placed in a conical glass vessel, and, after the excess of glycerine had been washed off with sterile saline, were pounded up by means of a glass rod. The pounded material was then emulsified in 20 c.c. of sterile saline. About 10 c.c. of this emulsion was diluted with saline until it could be drawn through a Berkefeld filter with the aid of an exhaust pump. The *Macacus rhesus* was injected with the unfiltered emulsion and the *patas* with the filtered.

SYMPTOMS AND LESIONS OF THE EXPERIMENTAL ANIMALS.

Briefly the effects produced were as follows:

(1) MACACUS RHESUS.

19/9/19.—Received $\frac{3}{4}$ c.c. of the unfiltered brain emulsion intra-cerebrally and 5 c.c. intra-peritoneally. This animal had been observed for some time before the experiment and was known to have a cough, but owing to the absence of others it was necessary to use it.

25/9/19.—Nothing abnormal to date.

2/10/19.—Tendency to be drowsy. This drowsiness gradually increased and the animal at times could only be roused by pulling its hair.

9/10/19.—Condition similar, but animal much weaker. No evident paralysis. Killed by chloroform.

Necropsy.—Subcutaneous fat very scanty. Brain pale ; slight increase of subdural fluid. Cord pale ; increase of cerebrospinal fluid, in which only a few lymphocytes found.

Lungs : Tuberculous broncho-pneumonia of right lung with a tuberculous abscess (tubercle bacilli present). Left lung, apart from slight œdema at the base, seemed normal.

The abdominal organs (liver, spleen and kidneys) appeared quite healthy. The mesenteric glands were slightly enlarged, and one or two showed commencing caseation.

The brain on section showed nothing abnormal, and pieces were removed and placed in 33 per cent. glycerine and in formaldehyde solution.

Histological examination.—Sections of the mid-brain, pons and cervical cord were examined. No inflammatory infiltration was present. In the mid-brain there were one or two small hæmorrhages. The nerve-cells showed some degeneration, but not sufficient to accept as proof of neuronic degeneration before death.

(2) PATAS MONKEY.

19/9/19.—Received 1 c.c. of the filtered emulsion intra-cerebrally and 5 c.c. intra-peritoneally.

25/9/19.—Monkey had been quite well until the forenoon of the 25th; it then had a fit. After a short interval the animal recovered, but was very tremulous and excited. The temperature was 102·2° F.

26/9/19.—Animal quite recovered.

9/10/19.—No further symptoms; animal appeared to be quite well.

6/11/19.—A severe fit in which the animal was almost rigid and lay at the bottom of the cage in a dazed condition.

7/11/19.—Animal much better; eat well, but unsteady on legs.

10/11/19.—Lay down in cage except when roused.

11/11/19.—Condition very similar, still considerable rigidity and trembling. Disinclined for food, and was frequently to be found with head bent, as if dosing, in a corner of the cage.

12/11/19.—Condition similar.

13/11/19.—Condition similar.

14/11/19.—Died in the afternoon. Placed in ice-chest over-night.

15/11/19.—*Necropsy.*—Nothing abnormal was found in any of the internal organs. Sections of the brain showed some congestion of the grey matter, but nothing of any diagnostic importance. Pieces of the cord, pons and basal nuclei were placed in 33 per cent. glycerine for inoculation purposes; the remainder of the central nervous system was placed in formaldehyde solution for histological examination.

28/11/19.—An emulsion of the cord and basal nuclei was inoculated into three monkeys (two *Macacus cynomolgus* and a *rhesus*), but as no effects were produced the animals, together with a *Cercopithecus callitrichus* monkey, were reinoculated fourteen days later. These animals are still under observation, and one of the *cynomolgus* monkeys has developed symptoms not unlike those of the *patas* monkey.

HISTOLOGICAL EXAMINATION OF THE PATAS MONKEY.

In order to obtain material for inoculation and rapid microscopic examination, a sagittal section was made through the brain, 1 cm. to the left of the great longitudinal fissure. From the left segment thus removed the left basal ganglia together with the greater part of the left centrum semiovale was scooped out. The greater part of the pons and a few portions of the spinal cord were also excised.

A horizontal segment of the left basal ganglia, a vertical segment of the upper part of the pons, and a segment of the cervical cord were immediately placed in 10 per cent. formaldehyde solution, and next day were embedded in paraffin. The remainder of the central nervous system was placed over-night in the ice-chest, was next day placed in 10 per cent. formaldehyde, and was later transferred to 10 per cent. saline formaldehyde.

The detection of specific changes in the horizontal section of the left basal ganglia led to an examination of the remainder of the central nervous system. In the following account of the microscopic findings the two portions removed for provisional examination are, for greater convenience, placed in sequence of situation among the portions examined subsequently.

The tissue was embedded in paraffin. Sections were stained in Ehrlich's hæmatoxylin with eosin, in Weigert's iron-hæmatoxylin with van Gieson's mixture, in Weigert's fuchselin with neutral red, by Gram's method with neutral red, in the Unna-Pappenheim mixture, and in Jenner's stain.

(1) *Right frontal pole.*—The vessels of the meninges are congested; many are partly occupied by hyaline thrombus. Around one or two vessels in the pia-arachnoid there is a very slight infiltration with lymphocytoid cells.

The vessels in the cerebral substance are also congested, and a few contain hyaline thrombus. The great majority of the pyramidal cells are slightly shrunken and deeply stained. The nucleus is stained deeply, and shows no internal structure apart from a nucleolus. It is central, or slightly eccentric. The cytoplasm is conspicuously dark. It is occupied by a deeply stained net, enclosing pale vacuolar spaces. Forming part of the net neuro-chromatic bodies can very occasionally be recognised. The net appears, therefore, to have been formed by the neuro-chromatin.

The remaining pyramidal cells are larger. The nucleus is large; the threads of chromatin are slender, feebly stained and separated so far as to form a widely meshed net; the nuclear membrane is very slender, pale and indistinct. The nucleus is central, or slightly eccentric. In the feebly stained cytoplasm is a widely meshed net of slender, slightly darker neuro-chromatin. In a very few cells neuro-chromatic bodies can be recognised as forming part of this net, where it occupies a dendron. Glial nuclei occasionally project into bays in the surface of both the dark and the pale cells.

(2) *Right, central, parietal cortex*, and (3) *Right occipital cortex.*—The changes are very similar, save that there is no meningeal infiltration. In the parietal cortex the deeply stained pyramidal cells do not preponderate so greatly over the pale; in the occipital cortex the pale cells are in excess of the deeply stained.

(4) *Coronal section through the left hemisphere in the anterior part of the frontal lobe.*—The pyramidal cells are almost all of the deeply stained type.

(5) *Coronal section through the left hemisphere at the level of the central parietal and temporal lobes.*—Hyaline thrombi in meningeal arteries and veins are numerous. There is a trace of infiltration with lymphocytoid cells in a few portions of the pia-arachnoid. Deeply stained pyramidal cells are not so numerous as in the previous section. In the pale cells traces of neuro-chromatic bodies are more frequent.

(6) *Coronal section through the left hemisphere in the region of the occipital lobe.*—Pale pyramidal cells are much more numerous than dark. In these pale cells evidence of degeneration is less, traces of neuro-chromatic bodies being more frequently present.

(7) *Horizontal section through the left basal ganglia and insula (segment removed for provisional examination).*—The section is at a low level. It includes the putamen and globi pallidi, part of the insular cortex, the external capsule, a large extent of the internal capsule, within which are small trabecular extensions from the head of the caudate nucleus, and a small portion of the subthalamic region.

In the posterior part of the convex outer border of the putamen there is a slight *infiltration* within, and immediately around, the adventitia of two large veins. A similar infiltration surrounds a capillary in the external capsule close to these veins.

In the subthalamic region several vessels show a similar, slight, intra- and peri-adventitial infiltration. In this region there is also a conspicuous, small, focal area of infiltration which does not surround a vessel.

The cells of the adventitial infiltrations measure from 5 to 6 micra in diameter. Their cytoplasm is basophil. Their nuclei measure about 4 micra in diameter, and contain numerous large deeply stained nodes. They are similar to the cells which are described in greater detail in Section 9.

The cells in the focal area of extra-adventitial infiltration have oval, and bent oval, nuclei, which in structure resemble those of glial cells.

No granular leucocytes were detected in the adventitial sheaths or in the glia.

Many of the glial cells throughout the section show karyorrhexis.

In the nerve cells of the caudate nucleus the cytoplasm is usually pale; neuro-chromatin is usually absent, but is sometimes present in the form of pale granules, or a fused ring, in the periphery of the cytoplasm. The nuclei are almost all pale, and show no structure save a nucleolus and a few scattered droplets of chromatin; rarely the nucleus is pyknotic.

The small cells of the putamen are pale. The cytoplasm contains no neuro-chromatin The nuclear membrane is seldom visible, and the nucleus is usually indicated only by a nucleolus and a few beads of chromatin.

In the large cells of the putamen and globi pallidi the nuclei are usually pyknotic. The neuro-chromatin usually forms swollen, often fused, masses, but it is sometimes powdered, sometimes confined to the periphery of the cells, and sometimes absent. A few of these large cells are very pale, vesicular, and without a nucleus.

The great majority of the cells in the subthalamic region have a pale, vesicular cytoplasm in which a few minute pale granules of neuro-chromatin are occasionally visible. A few cells have pyknotic nuclei and darker granules of neuro-chromatin.

(8) *Vertical section through the basal ganglia.*—The section includes the right basal ganglia and insula, the body of the left caudate nucleus, the inner six-eighths of the left optic thalamus, the optic chiasma and the corpus callosum.

The meninges, choroid plexuses and cerebral substance are congested. Hyaline thrombi are again present in many vessels. There are three small *hæmorrhages* into perivascular canals in the right globus pallidus, and two in the left ansa peduncularis.

In the mesial cortex of the right hemisphere the majority of the pyramidal cells are of the deeply stained type described above. This type of cell is less numerous in the insular cortex. The large cells in the right and left caudate nuclei, in the right pulvinar and globi pallidi, and in the nuclei of the optic thalami are less deeply stained. Their nuclei show a distinct structure. Their cytoplasm is usually vacuolated, and seldom shows a trace of neuro-chromatin. The periphery of the cytoplasm is frequently fragmented.

(9) *Section through the anterior extremity of the mid-brain.*—The section includes the pineal and mammary bodies, the cephalic extremity of the right anterior corpus quadrigeminum, the right pulvinar, the right internal and external geniculate bodies, the substantia nigra, and the anterior extremity of the oculomotor nuclei. On the right side the section extends to, and includes, the cerebral cortex. The left side is terminated laterally, at the outer margin of the crus, by the sagittal section which was made in order to obtain material for inoculation and provisional microscopic examination. The structures on the left side lie slightly nearer the cerebrum than on the right.

In the right lateral geniculate body the cytoplasm of the cells is devoid of neuro-chromatin, is vacuolated, and so fragmented in the periphery that the greater part of the nucleus frequently lies in an unoccupied portion of the perineuronal space. The cytoplasm of the cells in the median geniculate body is more deeply stained, less vacuolar and less fragmented. In the oculomotor nuclei a few cells are pyknotic. The majority are pale, their cytoplasm is structureless, their nucleus is frequently swollen and sometimes eccentric, whilst the periphery of the cell body is occasionally fragmented.

In the substantia nigra the neuro-chromatin is collected into large, often confluent, masses in the periphery of the cells. The nuclei are so pale, dropsical and ill-defined that they are difficult to detect. They are frequently eccentric.

In all the above sites glial nuclei frequently project into bays in the periphery of the nerve cells.

On the left side, at the edge of the coronal section, in the ventral portion of the optic thalamus, in a position immediately cephalic to the lateral geniculate body, is *a group of vessels surrounded by cellular infiltration.* Some of the infiltrating cells lie within the adventitial clefts or between the endothelium and adventitia, but the majority lie immediately external to the adventitia. The vessels are thus surrounded by conspicuous rings of infiltration. Relatively few infiltrating cells lie scattered through the glia at a distance from the perivascular rings. In this area degeneration of nerve-cells does not appear to be greater than in other parts of the section.

A capillary, immediately beneath the ventricular surface on the left side of the pineal body, is surrounded by similar infiltration.

The *cells* in the perivascular rings measure from 5 to 6 micra in diameter, the nuclei being about 4 micra in diameter. The cells are rounded, but one axis is usually longer than the other. The cytoplasm is decidedly basophil, but in the great majority of the cells is less basophil than that of a plasma cell. The nucleus is, as a rule, slightly eccentric. It contains conspicuous, large, deeply stained nodes on a widely meshed net of chromatin. Of these nodes one is frequently in the centre, whilst the remainder rest upon the nuclear membrane. Occasionally the nucleus is of the "clock-face" type, the nodes being triangular and resting with their bases upon the nuclear membrane. The latter nuclei resemble, therefore, those of plasma cells, but in the majority of these cells the cytoplasm is not so abundant and not so deeply basophil as in a typical plasma cell, whilst a pale perinuclear demilune is very seldom present.

In the infiltration of the glia, external to the perivascular rings, there are a few cells of the above kind; the majority of the cells differ in having a nucleus with the delicate structure of a glial cell. In a few perineuronic spaces are similar, free glial cells, and pyknotic, often shrivelled, nuclei devoid of cytoplasm (neuronophagia). In the glia are also portions of fragmented glial nuclei. One neutrophil leucocyte was found in the glia; no neutrophil leucocytes were found in the perivascular rings.

Almost the whole of the infiltrated area in this section is included in the field represented in Plate III. (Objective ⅔ inch; ocular Zeiss 4.)

(10) *Section through the anterior corpora quadrigemina and the right internal geniculate body.*—This section includes the nucleus and fibres of the third cranial nerves, the substantia nigra, the red nuclei and the upper motor nucleus of the trigeminus.

The large globular cells of the upper motor nucleus of the trigeminus are deeply stained and contain neuro-chromatic bodies.

In the oculomotor nuclei the cells show relatively little degeneration. They are of good shape and have central nuclei. The majority are pale, and contain a variable number of scattered, neuro-chromatic bodies. These bodies are usually globular. The other cells are smaller and deeply stained. In these the neuro-chromatic bodies are, as a rule, more numerous, are usually smaller, and of more regular triangular or fusiform shape.

In the red nuclei, also, there are both pale and dark cells. The majority are pale. In these pale cells neuro-chromatic bodies are usually scanty, swollen and globular. The dark cells appear to be shrivelled, and neuro-chromatic bodies can seldom be distinguished therein.

In the substantia nigra the neuro-chromatic bodies are confined to the periphery of the cells, and are usually fused. The nuclei are frequently slightly eccentric.

In the internal geniculate body almost all the cells are swollen, pale, vacuolated and devoid of neuro-chromatin.

The tissues are slightly separated by *œdema* in focal areas within the oculomotor nuclei, the red nuclei and the substantia nigra.

(11) *Vertical transverse section through upper part of the pons. (Segment removed for provisional examination).*—The section passes through the aqueduct of Sylvius and the caudal portion of the posterior corpora quadrigemina. In the grey matter close to the mid-line,

between the aqueduct and the dorsal longitudinal bundles, is a paired nucleus of large cells. This nucleus is apparently not the trochlear nucleus, but caudal thereto. The section includes transverse sections of the fibres of the trochlear nerve.

In the nerve-cells of the floor of the aqueduct and of the pons the neuro-chromatin forms ill-defined masses confined to the periphery of the cytoplasm, or impregnates the cytoplasm diffusely. The nuclei are central. In hæmatoxylin and eosin the staining resembles that of imperfectly fixed tissue. Many of the glial nuclei show karyorrhexis.

There is no evidence of inflammatory reaction.

(12) *Section through the anterior extremity of the medulla.*—The section includes the cerebellum. In this there are three nuclei on each side; on analogy with the cat these are the dentate, the combined embolus and nucleus globosus, and the nucleus tecti. Beneath the fourth ventricle is the dorsal vestibular nucleus and a small nucleus mesial thereto. Ventral and slightly lateral to the vestibular nucleus lies a nucleus composed of large cells, apparently Deiter's nucleus. Lateral to this is a second nucleus of large cells, apparently the nucleus of Bechterew. A small portion of the olives is included. Mesial to this, and slightly ventral, above the dorsal border of the trapezoid body, is a small nucleus, probably the accessory nucleus of the sixth cranial nerve.

The majority of the Purkinje cells are large and pale, and contain only a few scattered, usually rounded, neuro-chromatic bodies. The remaining cells are stained deeply; the larger examples have a deeply stained cytoplasm in which neuro-chromatic bodies cannot be recognised; a few smaller examples contain numerous neuro-chromatic bodies of spindle shape.

The cerebellar nuclei also contain both deeply stained and larger, pale cells. In both forms neuro-chromatic granules are almost invariably absent. The pale cells preponderate.

In the dorsal vestibular nucleus, Deiter's nucleus and Bechterew's nucleus and the accessory sixth nucleus, the bodies of the neurons show similar changes, but scattered neuro-chromatic bodies are more frequently seen.

The majority of the cells of the olives contain deeply stained neuro-chromatic bodies; in some cells these bodies are swollen and rounded; in a few they are absent in a perinuclear zone.

In the accessory sixth nuclei, especially in that on the left side, the glia is dissociated by *œdema.*

In the trapezoid body, above the outer extremity of the right peduncle, *a capillary is infiltrated* by cells similar to those seen in sections 7 and 9. The infiltrated cells lie between the endothelium and the adventitia. This vessel is represented in Plate IV. (Objective $\frac{1}{12}$ inch, ocular Zeiss 4.)

(13) *Section through the posterior extremity of the medulla.*—The section includes the following structures: The internal arcuate fibres; three bilateral large dorsal nuclei separated by zones of longitudinal fibres—the mesial of these is obviously the gracile nucleus, the external has very large cells and is presumably the cuneate or accessory cuneate, whilst the third nucleus lies between and slightly ventral to these, and has cells similar to those of the gracile nucleus; the nuclei of the central grey matter; the separated ventral portion of the anterior horn of the cord (nucleus ambiguus); the olives, and mesial olives.

In the gracile nuclei there are a few pyknotic cells on the left side. The remaining cells are pale and swollen; neuro-chromatin is confined to the extreme periphery of the cells in the form of a few small bodies; the cytoplasm is often greatly vacuolated and the nucleus completely lysed. The cells of the intermediate dorsal nucleus show similar changes.

In the lateral dorsal nucleus (cuneate nucleus) neuro-chromatin is in general more abundant, but where most abundant it is scanty and is in the form of rounded bodies of different sizes.

In the central grey matter and in the nucleus ambiguus the cells are pale. Their nuclei are slightly dropsical. Neuro-chromatin is either absent whilst the cytoplasm is vacuolated, or it is confined to the periphery of the cells in small granules or in swollen clumps.

In the olives the neuro-chromatolysis is in general at its least. But it is present in different degrees, and several cells have lost their nuclei. In the median olives the neuro-chromatolysis is greater.

(14) *Upper cervical cord.*—A few of the cells of the anterior horns contain neuro-chromatic bodies of fairly good shape and distribution. The great majority of the cells are swollen, vacuolated and pale, have a dropsical nucleus, and contain at the most only a few, ill-defined, poorly stained particles of neuro-chromatin. In many cells the peripheral cytoplasm is fragmented by vacuoles; in these cells the nucleus is very difficult to distinguish or cannot be seen. Shrunken pyknotic cells with bays in their periphery are scanty. In the posterior horns pyknotic cells are relatively abundant.

(15) *Cervical enlargement.* (*Segment removed for provisional examination.*)—The sections include a portion of a posterior root ganglion.

The nerve-cells have been fixed imperfectly, but neuro-chromatic bodies can be demonstrated throughout their cytoplasm.

(16) *Cervical enlargement.*—The section includes the nerve roots.

Degenerations similar to those in Section 14 are present. but there are many more cells containing neuro-chromatin. In several cells the neuro-chromatic bodies are deeply stained and evenly distributed.

(17) *Upper thoracic cord.*—Degeneration is greater than in the preceding section. Shrinkage of the neurons, with crenation of their outer borders, is commoner than swelling and vesiculation.

(18) *Mid thoracic cord.*—In the anterior and lateral horns the changes are very similar. In Clark's column the cells contain numerous neuro-chromatic bodies.

(19) *Lumbar enlargement and roots.*—There is a great difference in the appearance of different neurons. All the types of degeneration already described are present. Some cells are obviously swollen; their neuro-chromatin is represented by ill-defined bodies in the extreme periphery of the cytoplasm; their nuclei are eccentric, and the nucleolus therein may lie against the nuclear membrane. Such a cell often lies next a slightly shrivelled cell containing swollen, deeply stained neuro-chromatic bodies. Where neuro-chromatin is abundant, it is almost invariably in the form of bodies of different size and shape.

(20) *Sacral cord with cauda equina.*—Less degenerate cells with evenly distributed neuro-chromatic bodies are more frequent in this segment, but many cells show neuro-chromatolysis, vacuolation, disintegration or pyknosis.

(21) *Lung.*—There is slight anthracosis. The bronchi and bronchioles are healthy. There is catarrhal desquamation of epithelial cells in a few alveoli which lie against bronchioles. There are also a few small areas of collapse. The remainder of the vesicular tissue shows emphysematous distension.

No bacteria are present.

(22) *Liver.*—The liver is engorged.

(23) *Kidney.*—The kidney is engorged. The epithelium of the tubules shows slight cloudy swelling. There are no casts. Active inflammatory reaction is absent.

(24) *Spleen.*—There is a remarkable quantity of iron (Prussian blue reaction) in the form of coarse granules, not only in the pulp but in Malpighian bodies. There is no evidence of inflammation.

Examination of central nervous system for bacteria.—No bacteria were detected within the substance of the tissue or within the vessels. Bacteria were, however, found upon the surface of segments taken from the material which had been placed in the ice-chest overnight before fixation. They were present upon the surface of segment 9, including the surface of the coronal section made by the knife in separating the left cerebral hemisphere. They were also present upon the outer surface of the dural theca of the spinal cord, and of nerve roots outside the theca. The majority of the bacteria were Gram-negative bacilli. With these were associated Gram-negative diplococci.

In this monkey, therefore, no pathological changes of significance outside the central nervous system were found on examination with the naked eye and with the microscope.

Within the central nervous system active inflammation was present in the posterior part of the left basal ganglia and in the junction of the left basal ganglia with the mid-brain; inflammation represented by a very slight perivascular infiltration of the pia was present over areas of the cerebrum; an inflammatory infiltration of the wall of one vessel was also present in the trapezoid body on the right side of the anterior extremity of the medulla.

The changes other than inflammatory infiltration were: engorgement, hyaline thrombosis, hæmorrhages into "perivascular canals" in the right globus pallidus and left ansa peduncularis, abnormalities in the bodies of neurons, and œdema of certain cranial nuclei. The exact significance of the abnormalities in the bodies of the neurons is very difficult to determine. It must be borne in mind that the animal was found dead in its cage, part of the material was hurried through fixation, and the remainder was left in the ice-chest for two nights before fixation. Bacteria were present upon the exposed surfaces of the latter material. Under these circumstances the bodies of the neurons and their neuro-chromatin could not be expected to be in perfect condition. The severer forms of ante-mortem degeneration and necrosis were not detected. With few exceptions the degenerations were similar to those which have been found in material fixed in formaldehyde solution after removal from cases in which death had been due to accident and the cadavera had been preserved over-night in the freezing-chambers. But there were exceptions. For instance, the degeneration of some of the cells of the anterior horns of the lumbar cord cannot be ascribed to changes after death. Further, these cells were in close proximity to others in which little, if any, degeneration could be detected. Such divergence in the condition of cells in particular nuclei was common, and is in favour of degeneration before death. It can only be said that there was evidence of the occurrence of neuronic degeneration before death, but that the degenerations actually observed cannot be accepted in their entirety as having occurred before death. The cells in which the degeneration appeared to be greater than could be attributed to changes after death, were distributed throughout the central nervous system.

The focal œdematous dissociation of the tissues in certain nuclei cannot be attributed to changes after death. Further, such focal œdema is very common in these very nuclei when infiltrated in infantile paralysis and *encephalitis lethargica.*

CONCLUSIONS.

There can be but little doubt that, in this experiment, *encephalitis lethargica* developed in a monkey after the inoculation of the filtered emulsion from the brain of a human case of this disease. The clinical manifestations in the monkey resembled with considerable closeness those of human *encephalitis* *lethargica.* The histological changes reproduced in exaggerated form the chief characteristics of human *encephalitis lethargica*: inflammatory infiltration was very strictly localised, and was confined to the areas chiefly affected in the human disease. The finer histological features were also reproduced: the grosser forms of cellular necrosis were not found; neurono-phagia was very slight; a search with special methods for emigrated granular leucocytes revealed only one such cell.

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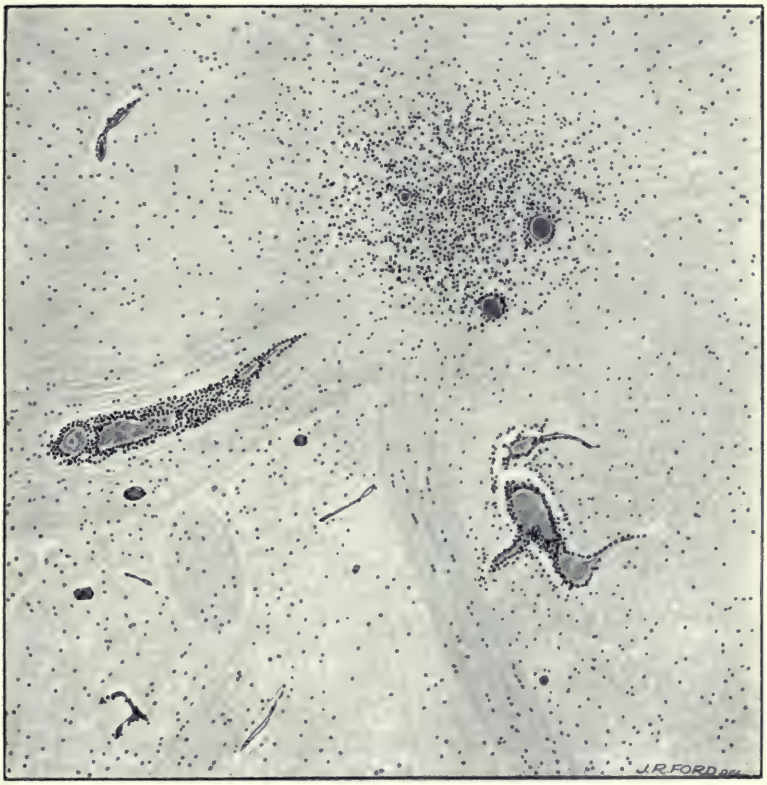
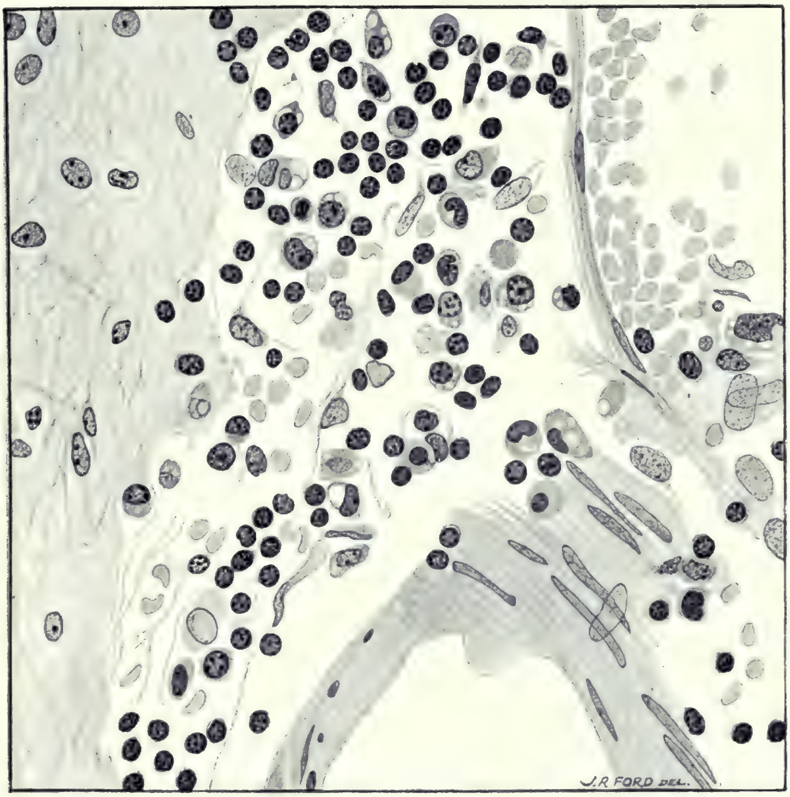


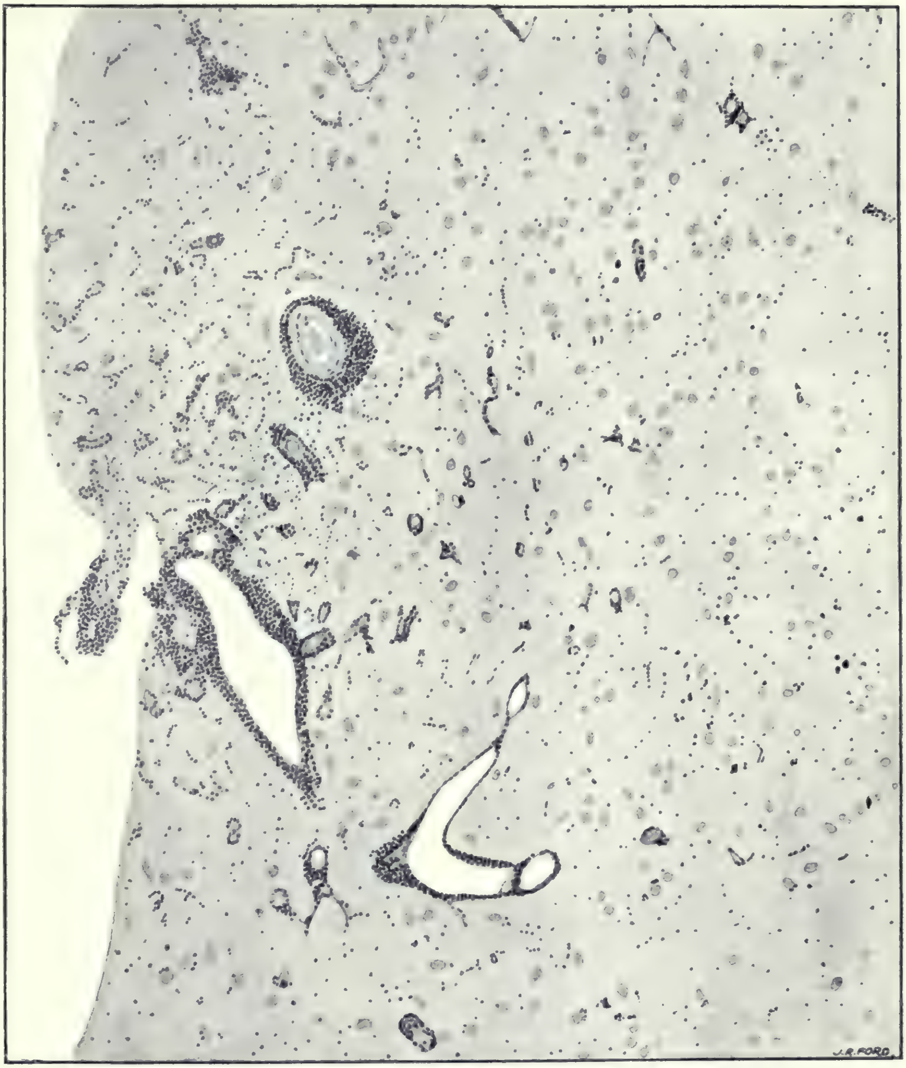
Human case for comparison with Plate III. Man, aged 48 years.
Basal nuclei. Weigert's iron-hæmatoxylin and acid fuchsin.
Obj.: $\frac{2}{3}''$. Oc.: Zeiss 4.

Plate I.

McIntosh and Turnbull. Lethargic Encephalitis.

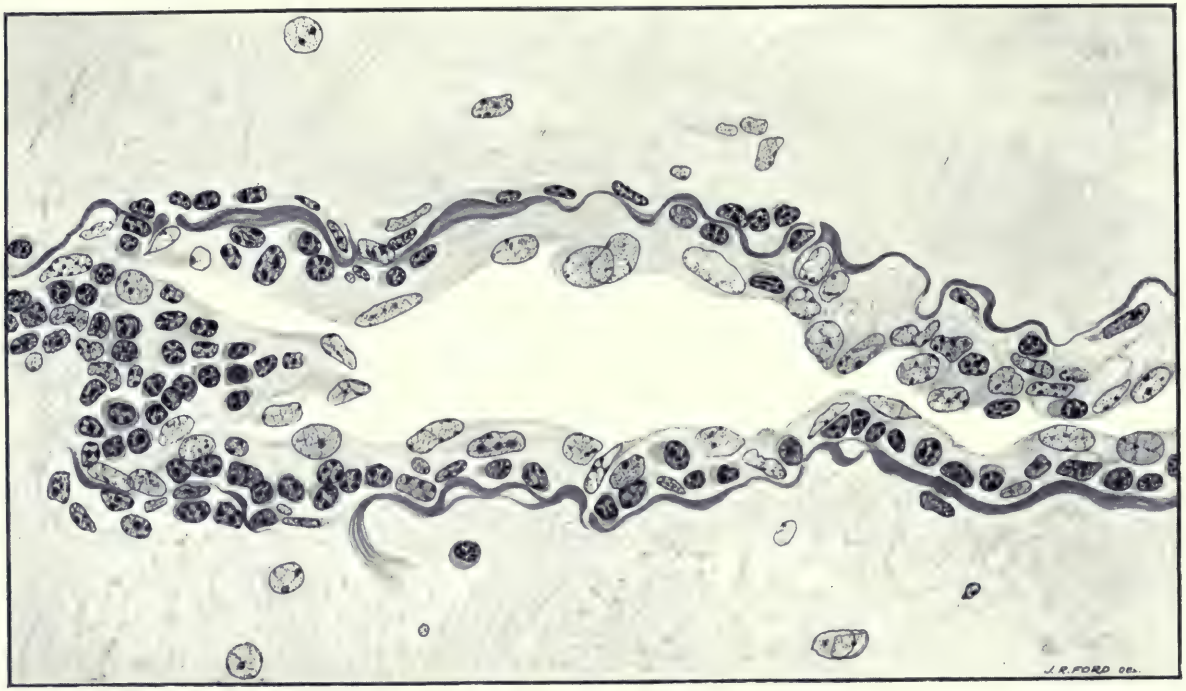


Human case for comparison with Plate IV. Man, aged 48 years. Basal nuclei. Weigert's iron-hæmatoxylin and acid fuchsin. Obj.: 3 mm. Oc.: Zeiss 4.



Patas monkey. Left optic thalamus (Section 9, p. 98). Jenner's stain. Obj.: $\frac{2}{3}''$. Oc.: Zeiss 4.

Plate III.



Patas monkey. Right trapezium (Section 12, p. 99). Weigert's iron-hæmatoxylin and van Gieson's mixture.
Obj.: $\frac{1}{12}$″. Oc.: Zeiss 4.

Plate IV.

CONDITIONS WHICH ARE CONDUCIVE TO THE PRODUCTION OF SHOCK BY HISTAMINE.

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DETAILS of the shock-like collapse of the circulation, associated with depression or failure of the respiration, which is produced by histamine in anæsthetised cats and dogs, have been published by P. P. Laidlaw, A. N. Richards and myself (1, 2, 3, 4). The central feature of the condition appeared to be an oligæmia, a diminution of the volume of blood in effective circulation, which we were led to attribute to a loss of the normal tone of the capillary blood-vessels, and a morbid permeability of their endothelial walls. We were interested in this action, produced by an organic base, not merely as a pharmacological curiosity, but because it appeared to represent a type of action common to a large group of poisonous substances of animal or bacterial origin. It had, at the time of our publication, an immediate interest in its resemblance to a shock-like condition prevalent during the war as a sequel to massive injury of the tissues, in which an aseptic toxæmia appeared to be a prominent factor (Cannon and Bayliss, 5), (Bayliss, 6, 7), (Cannon, 8, 9), (Quénu, 10), (Delbet, 11). We believed, moreover, that the type of action would have a more general pathological interest, in that it represented a generalisation over the whole circulatory system of phenomena recognised, when occurring locally, as inflammation, and had therefore an important bearing on the mechanism of the failure of circulation and depression of metabolism associated with many types of rapidly generalised infection or toxæmia.

There has been evident, in certain more recent publications, a tendency to attribute to the resemblance in action a chemical significance, for which we found, and for which I still find, insufficient justification. Histamine, of course, is closely related to and easily derived from the amino-acid histidine; and an attractive simplification could be effected in our conception of many pathological processes if we could assume that, wherever an action of this type is detected, histamine itself has been produced. Abel and Kubota (12), in a highly suggestive paper, have given evidence which leads them to the conclusion that histamine is produced with great readiness from muscle, liver and other tissues, and is split off at an early stage in the hydrolysis of any ordinary protein. On the chemical side, however, the evidence is still incomplete, and the suggestion that histamine itself, or even a derivative of histamine, is actually formed when a tissue is injured can only be regarded as an open possibility. In dealing with certain influences which have an adjuvant effect on the poisonous effect of histamine, I am, therefore, still regarding this substance simply as a convenient agent for the quantitative production of a certain type of effect. I have already published, in brief summary, a note on some of the factors which contribute to the production of histamine-shock (4). My purpose here is to give a fuller account of the experiments there mentioned and of some others, and to discuss their practical implications.

EFFECT OF ANÆSTHETICS.

Laidlaw and I (2) mentioned the fact that anæsthetisation of the animal (usually a cat) by chloroform, ether or urethane, or by decerebration under a preliminary volatile anæsthetic, was a factor in the production of histamine-shock. We had observed the toleration of relatively very large doses of histamine by unæsthetised cats, and had been led to relate the occasional failure of the shock to appear, in cats under ether, to the rapidity of the preliminary preparation, the animal having been only a short time under the anæsthetic before the dose of histamine was injected. We had not, however, deliberately explored the extent of the tolerance in the normal animal, nor had we eliminated the factor of individual variation of resistance. In the following experiments this has been effected by testing the effect of histamine on the same cat under different conditions, with an interval of some days between the two experiments, to exclude any after-effect of the first:

Cat 1.—February 5th. Experiment without anæsthesia. Weight 2300 grm. Paws unpigmented. The animal was gently secured on a warmed table. A drop of blood from the ear-vein had a hæmoglobin value of 88 per cent.

12.14 p.m.—Begin slow infusion of 0·1 per cent. histamine into the left internal saphena vein.

12.16 p.m.—Cat salivating. Heart-beat slow (vagus inhibition), respiration deep. 1 mgrm. injected.

12.19 p.m.—3 mgrm. injected. Heart still inhibited by vagus, but beats vigorous. Paws and buccal mucosa bright pink.

12.22 p.m.—5 mgrm. injected. Pupils widely dilated.

12.24 p.m.—6 mgrm. injected. Mucous membranes slightly dusky. Heart-beat quicker.

12.28 p.m.—10 mgrm. injected. Condition much the same. Respiration more rapid. Pupils still dilated.

The infusion was continued at about the same rate, with little change in the condition, till 12.43 p.m., by which time 23 mgrm., *i. e.* 10 mgrm. per kilogram, had been injected. The infusion was then stopped and the cat released. It seemed a little stiff in its movements, but stood and walked. Respiration was not perceptibly abnormal and the heart-beat was vigorous. A sample of blood from the ear-vein at 12.50 p.m. showed a hæmoglobin value of

98 per cent., indicating some concentration. The cat soon began to clean its fur, jumped from the table and ran across the room. There was no attempt at vomiting during the infusion; fæces had been passed, but without violent purgation. At 2 p.m. the animal still seemed to be perfectly normal, and a third sample of blood showed return to the initial hæmoglobin value of 88 per cent.

The cat remained in perfect health till February 10th, when the second experiment was performed. The weight was still 2300 grm.

Cat 1.—February 10th. Experiment under ether anæsthesia maintained for two hours before histamine was given.

2.45 p.m.—Cat anæsthetised with chloroform and then kept under light anæsthesia with ether till 4.37 p.m.

4.37 p.m.—Rectal temperature 36·8° C. Ether strengthened till anæsthesia was deep. Tracheal cannula inserted, through which ether was then given. Arrangements for recording arterial pressure and taking samples of arterial blood. Both carotids tied and both vagi cut.

4.54 p.m.—Start record. Blood-pressure 190 mm. Hæmoglobin 75 per cent.

5.2 p.m.—Blood-pressure 160 mm. Hæmoglobin 75 per cent.

5.5–5.6 p.m.—Inject 2·3 mgrm. of histamine (1 mgrm. per kilo.) into femoral vein. Blood-pressure falls rapidly from 160 mm. to less than 40 mm. (see Fig. 1). When the blood-pressure reaches about 45 mm. respiration fails. Artificial respiration applied and continued thereafter.

5.12 p.m.—Inject a further 2·3 mgrm. of histamine, making 2 mgrm. per kilo. in all. The blood-pressure shows a slight temporary rise to 40 mm., and then falls further, the output of the heart becoming so small as scarcely to move the mercury in the manometer.

5.16 p.m.—Blood-pressure 26 mm. Hæmoglobin 118 per cent.

5.24 p.m.—Blood-pressure about 20 mm. Output practically imperceptible.

Thereafter, till the animal was killed at 6.12 p.m., the arterial pressure remained at about 18 mm. The beat of the heart, as felt through the chest-wall, was fairly vigorous, but the output was so small as to cause no visible pulse-waves on the manometer record. By compressing the abdomen, and temporarily driving blood into the heart, the pressure could be raised to about 45–50 mm., and the pulse at once became visible on the record. On relaxing the compression the circulation relapsed to its previous condition.

The post-mortem appearances were typical of histamine-shock as described in our earlier papers. The heart was practically empty.

The contrast between the effects of histamine on the same cat, under the different conditions of the two experiments, was very striking. In the case in which no anæsthetic was administered 10 mgrm. per kilo. were tolerated with but slight and evanescent effects; at the end of two hours under ether, although the animal was apparently in excellent condition, with an arterial pressure of 160 mm. after section of the vagi, 1 mgrm. of histamine per kilo. produced a profound condition of shock, and after 2 mgrm. per kilo. all tendency to recovery vanished. Several other experiments were made in which histamine was similarly infused into a vein without anæsthetic, and the results were, in general, the same. One further such experiment may be summarised.

Cat 2.—Weight 2100 grm. Blood from ear-vein has hæmoglobin value 72 per cent Respiration 56 per minute. 21 mgrm. of histamine (10 mgrm. per kilo.) were injected in 45 minutes. The pupils were dilated in the early stages, and the respiration deep. After 5 mgrm. had been injected the mucous membranes were slightly blue, and the respiration very rapid (140 per minute) and shallow. Thereafter the cat became distinctly narcotised, the pupils gradually contracted, and the breathing became slow and quiet. If the cat was roused deliberately it panted rapidly with open mouth, but relapsed into stupor with quiet breathing. When the injection was finished the animal lay as if asleep, and could not be roused and made to stand. The hæmoglobin value had risen to 92 per cent. Within an hour the cat had regained its normal activity and the hæmoglobin was 82 per cent. After another hour the animal had perfectly recovered and the hæmoglobin value had returned to the initial 72 per cent.

No experiments were tried with larger doses than 10 mgrm. per kilo., but there seems little doubt that this limit could have been exceeded without fatal or even serious effect.

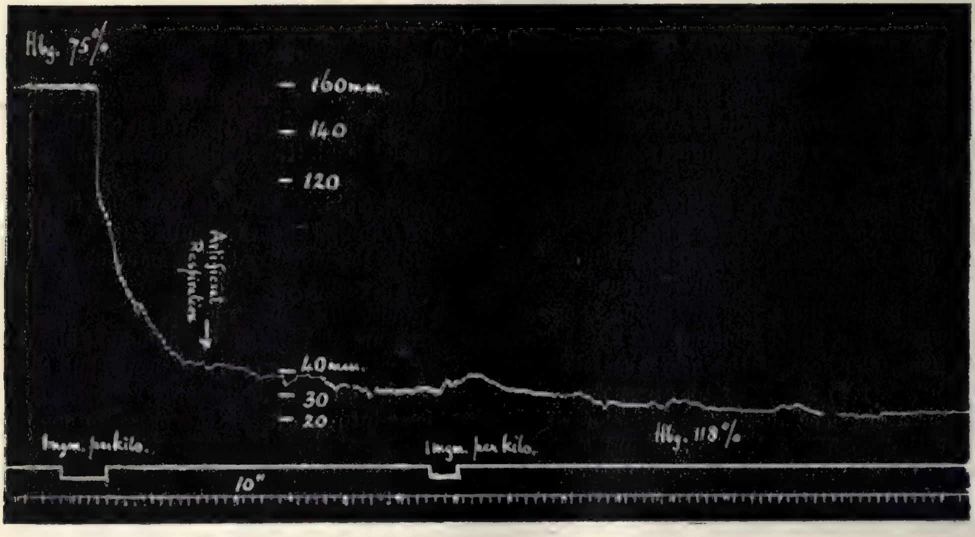

Fig. 1.—*Cat* 1. Effect of 2 mgrm. of histamine per kilo. on arterial pressure, at the end of two hours' anæsthesia with ether.

The experiment on Cat 1 after two hours under ether having been made during continued ether anæsthesia, it seemed desirable to determine whether the effect of the anæsthetic persisted after its removal.

Cat 3, weighing 4400 grm., was therefore kept for 2 hours under light ether and then allowed to recover. Thirty-five minutes after the administration of ether was discontinued the animal was still slightly drowsy, but otherwise normal. 9 mgrm. of histamine (*i.e.* just over 2 mgrm. per kilo.) were then infused into the saphena vein in 15 minutes. Cyanosis of the mucous membranes appeared almost as soon as the infusion was started. When it was finished the animal lay deeply unconscious, with shallow, rather rapid respirations, occasionally interrupted by a deep inspiratory gasp. As there was no sign of recovery a little ether was given —very little sufficing to abolish the corneal reflex completely—tracheotomy was performed, and the arterial blood-pressure recorded. It was about 100 mm., rising to 130 mm. after a deep inspiration, but the pulse was hardly perceptible on the record. The pressure gradually fell away, and spontaneous respiration failed. Artificial respiration was applied, but did not arrest the fall of blood-pressure, which reached 60 mm. after about 20 minutes. On opening the abdomen the typical appearance in histamine-shock was found—the intestines dusky, with dark venules, the pancreas purple and œdematous, the large veins poorly filled. The heart, though beating strongly, was very empty, and on incision ejected only a few c.c. of blood.

A sample of arterial blood showed a hæmoglobin value of 146 per cent. and a corpuscular content, by hæmatocrit, of 64 per cent. An initial control sample had unfortunately not been taken, but, even if the normal hæmoglobin value were over 90 per cent., which is very unusual in the cat, and the corpuscular content 40 per cent. instead of the average 33 per cent., the concentration caused by histamine would have been of an extreme order.

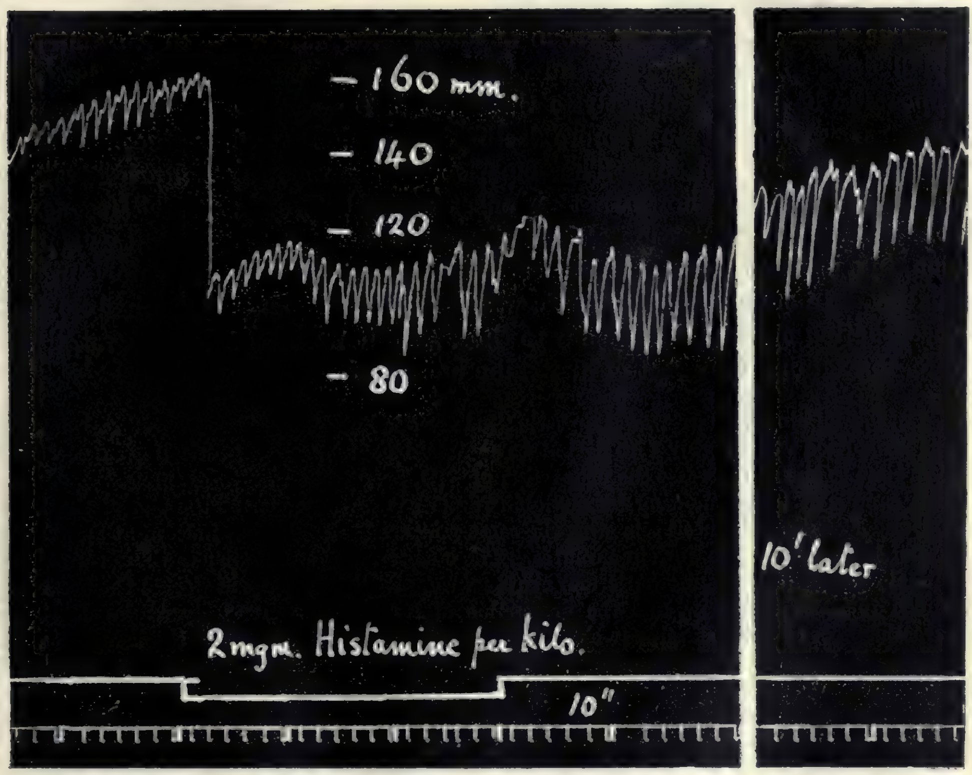

Fig. 2.—*Cat* 7. Effect of 2 mgrm. of histamine per kilo. on arterial pressure, at the end of one hour's anæsthesia with nitrous oxide and oxygen.

It is clear, then, that the adjuvant effect of ether on the shock-producing action of histamine persists after most of the ether has been eliminated from the system.

In another experiment a longer interval of recovery after ether was allowed.

Cat 4, weighing 3200 grm., was kept under light ether for two hours, and then left for two hours, when it had apparently recovered completely. 12·8 mgrm. (*i.e.* 4 mgrm. per kilo.) of histamine were then infused into the saphena vein in eighteen minutes. At the end of the infusion the animal was deeply narcotised, the limbs were rigid, and the respiration took the form of rapid panting, with open mouth and protruded tongue. The hæmoglobin value had risen from the initial 84 per cent. to 96 per cent.

The cat lay unconscious for half an hour, but was sitting up at the end of an hour. The hæmoglobin value was still 96 per cent., and even two hours later the animal still seemed drowsy and weak, and the hæmoglobin value was 98 per cent.

By the next day this cat had completely recovered; but it is clear that the preliminary period under ether, even though its obvious effects had passed off, had so lowered the resistance to histamine that 4 mgrm. per kilo. produced a much profounder and more lasting depression than 10 mgrm. per kilo. usually produces in the perfectly normal cat.

The effect, then, of this group of anæsthetics, under which histamine in small doses produces shock, and of which I have taken ether as the type, appears to be due not directly to the effect of the anæsthetic on the central nervous system, but to a more lasting depression of some mechanism of resistance to the poison. The experience of surgeons at clearing-stations during the war had shown that an onset of fatal "wound-shock" frequently followed the administration of chloroform or ether for some necessary operation, in a patient whose general condition had previously given no cause for anxiety. In such cases anæsthesia with nitrous oxide and oxygen had no such accentuating effect on a tendency to shock, and it was of special interest, therefore, to examine the effect of histamine under this anæsthetic.

Cat 5, weighing 3500 grm., was anæsthetised for one hour with nitrous oxide and oxygen. The proportion was roughly 4 to 1, but to maintain complete anæsthesia it was necessary from time to time to increase the proportion of N_2O, producing a slight cyanosis.

A sample of blood, showing hæmoglobin 78 per cent., was taken at the end of the hour, and under continued anæsthesia with the same mixture histamine was infused at an average rate of 1 mgrm. per kilo. in four minutes. The effects were very similar to those seen in the animal without general anæsthetic. When 2 mgrm. per kilo. had been injected the breathing tended to become shallow, and the proportion of N_2O was progressively weakened as the narcotic effect of the histamine itself became pronounced. By the end of the infusion, when the cat had received 35 mgrm. (= 10 mgrm. per kilo.) in forty minutes, equal parts of nitrous oxide and oxygen were being given.

Reflexes returned almost immediately on removing the administration mask, but the cat was unable to stand. The hæmoglobin value was 100 per cent. An hour later, when the cat could walk, it was still 100 per cent., and two hours later, when recovery seemed otherwise complete, 96 per cent.

Next morning the cat was perfectly well. It had eaten heartily during the night and the hæmoglobin value was 74 per cent. It was again anæsthetised, this time with ether, for a preliminary period of one hour. Then, still under ether, arrangements were made to record the arterial pressure and infusion of histamine begun. By the time 5 mgrm. (= about 1·4 mgrm. per kilo.) had been injected in seven minutes the blood-pressure had fallen from the initial 186 mm. to 44 mm., and the respiration had failed altogether. Under artificial respiration a further 2 mgrm. (making a total of 7 mgrm., or 2 mgrm. per kilo.) reduced the blood-pressure to 41 mm. Hæmoglobin value 106 per cent. After about fifteen minutes a slow recovery of blood-pressure, under continued artificial respiration, became perceptible, and it stood at 60 mm. half an hour after the injection was stopped. It was again reduced to 35 mm. by injection of 1 mgrm. of histamine. On opening the chest the characteristically empty but strongly beating heart of histamine shock was seen. The intestines were duskily congested, the pancreas œdematous. The heart was cut open and the experiment finished.

It will be seen that in this cat 10 mgrm. of histamine per kilo., administered during prolonged anæsthesia with nitrous oxide, produced but little more effect than that produced by the same dosage without anæsthesia; under ether, 2 mgrm. per kilo. produced an intense shock, which would have been immediately fatal but for the application of artificial respiration.

Further experiments were made under nitrous oxide and oxygen, in which the arterial pressure was recorded from the carotid artery.

Cat 6.—Weight 1600 grm. Anæsthesia with N_2O and O_2 for thirty-eight minutes before record started. Blood-pressure 165 mm. Hæmoglobin 74 per cent. 16 mgrm. of histamine injected intravenously in less than twelve minutes. Blood-pressure falls slowly to 100 mm.; then, after 4 mgrm. have been injected, slowly recovers. At the end of the infusion it is 150 mm. Hæmoglobin 86 per cent. Fifteen minutes later blood-pressure 140 mm. Cat killed under anæsthesia.

Cat 7.—Weight 2900 grm. Kept under N_2O and O_2 for an hour before beginning the record of arterial pressure (reproduced in Fig. 2). Vagi cut. Blood-pressure, fluctuating rather widely with respiration, about 160 mm. Hæmoglobin 84 per cent.

Inject 6 mgrm. of histamine (= about 2 mgrm. per kilo.) in three minutes. Blood-pressure falls immediately to about 110 mm.; three minutes later still 110 mm.; hæmoglobin 82 per cent.; ten minutes later blood-pressure 130 mm. Cat killed under anæsthetic.

The contrast between the effects of histamine, under ether and under nitrous oxide respectively, will be sufficiently obvious from a comparison of Figs. 1 and 2. Note that in Cat 7, 2 mgrm. of histamine per kilo. produced no increase in the concentration of the blood.

EFFECT OF PRELIMINARY HÆMORRHAGE.

A large proportion of the sufferers from wound-shock had probably had more or less serious hæmorrhage at the time of the infliction of the wound. The amount of blood lost would seldom be on record, and a calculation based on the corpuscular content of the remaining blood would be vitiated by the tendency of the shock-factor to produce concentration, in place of the normal dilution following simple hæmorrhage. The corpuscular content might seem surprisingly normal, and was often reported as doing so; but the initial hæmorrhage, though its effect on the blood was thus masked, would obviously intensify the severity of an oligæmia subsequently produced by loss of plasma into the tissues and stagnation of blood in the capillaries. Experiment showed that a preliminary hæmorrhage greatly weakened the resistance of the unanæsthetised animal to histamine.

Cat 8.—Weight 2100 grm. The left jugular vein was punctured and about 50 c.c. of blood withdrawn. The hæmoglobin value fell from 86 per cent. to 60 per cent. The cat seemed drowsy and lethargic, but otherwise normal. Two and a half hours later the hæmoglobin was still 60 per cent. Infusion of histamine was then begun into the saphena vein at the rate of 1 mgrm. in about one and a half minutes. The respiration was almost immediately affected and became progressively shallower. When 6 mgrm. had been injected in ten minutes the infusion was stopped. The hæmoglobin had risen to 74 per cent. The respiration continued to get weaker and failed altogether seven minutes after the infusion was stopped. The heart could still be felt beating vigorously. *Post-mortem* the typical picture of fatal histamine shock was presented.

The preliminary hæmorrhage had so weakened resistance that rather less than 3 mgrm. of histamine per kilo., injected without general anæsthesia, sufficed to produce fatal shock.

EFFECT OF REMOVING THE SUPRARENAL GLANDS.

Richards and I (1) drew attention to the possibility of an antagonism between adrenaline, on the one hand, and histamine and the group of capillary poisons with similar action, on the other. We found that in the artificially perfused organ the capillary tone which histamine relaxes could only be maintained if the perfusing blood contained a trace of adrenaline; so that the presence of the latter was necessary for demonstration of the vasodilator effect of histamine in an artificial perfusion. We suggested that a normal function of adrenaline might be the maintenance of capillary tone against the depressant action of traces of the products of cellular injury or metabolism. It is consistent with that suggestion, though not decisive evidence in its favour, that the cat deprived of its suprarenal glands shows a remarkable lack of resistance to histamine. In these experiments the suprarenal glands were removed with aseptic precautions under ether. Particular care was taken to avoid hæmorrhage and to secure absolute hæmostasis before the wound was closed. On the following day, when the animal had completely recovered from the immediate effects of the anæsthetic, and before serious symptoms due to suprarenal defect had made their appearance, histamine was infused intravenously without anæsthesia. In the earlier experiments, when the extreme sensitiveness to the drug was not expected, a dose probably in excess of that needed for a fatal effect had been injected before the result was realised. The following table shows the doses after which death occurred.

Weight of cat.	Time of decapsulation.	Time of injection of histamine on following day.	Dose after which death occurred.
Cat 9, 2000 grm.	4 p.m.	2.33 p.m.	1·5 mgrm. (= 0·75 mgrm. per kilo).
Cat 10, 2100 „	6 p.m.	11.21 a.m.	1·5 „ (= 0·71 „ „).
Cat 11, 3000 „	11.45 a.m.	9.50 a.m.	0·5 „ (= 0·16 „ „).
Cat 12, 2500 „	12 noon.	12 noon.	1·3 „ (= 0·5 „ „).

Cats 11 and 12 had each, some days before operation, received an injection of 10 mgrm. histamine per kilo. with the usual evanescent symptoms and complete recovery.

Another cat (13) was decapsulated under ether, kept till the following day, and then anæsthetised with ether again and the blood-pressure recorded. The blood-pressure was about 80 mm., the pulse slow, and the beat of the heart, as felt through the chest-wall, vigorous. On section of the vagi the beat became rapid and the pulse waves recorded by the mercury manometer so small as to be almost invisible. On tying the other carotid artery the pressure rose to 90 mm. and then gradually sank to 58 mm. The picture suggested the presence already of an incomplete "shock" of the histamine type. Injection of 0·01 mgrm. of histamine caused a fall of pressure to 35 mm. There was but slight tendency to recovery from this minute dose, the effect of which in the normal animal is very evanescent. The arterial pressure returned only to 42 mm., respiration failed, and in spite of artificial respiration the pressure gradually fell to 18 mm. Though the manometer recorded practically an unbroken straight line, the heart could be felt beating strongly through the chest-wall, but gradually became weaker and failed altogether 8 minutes later. It was found to be practically empty.

There seems to be a resemblance between the condition of the circulation, which slowly develops after the suprarenal glands have been extirpated, and that which histamine rapidly produces in the animal under ether. The resemblance is emphasised by the appearance of a condition which I have not elsewhere found recorded, but which I have observed in all cases in which I looked for it, namely, the pronounced concentration of the blood which occurs during the period of survival after extirpation of the suprarenal glands. Thus in Cat 11 the hæmoglobin value of blood from the ear-vein rose from 72 to 96 per cent., in Cat 12 from 76 to 104 per cent., in the period of about 24 hours between the extirpation and the fatal injection of histamine. In Cat 13 it was initially 82 per cent., 98 per cent. 24 hours later, and 109 per cent. after administration of ether for the record of blood-pressure, but before giving histamine. All the operations were performed extra-peritoneally, through lumbar incisions, and the appearance of extensive œdema in the retro-peritoneal tissues, discovered *post-mortem*, to some extent accounts for the loss of blood-plasma. The concentration was not proportionate to the extent of this œdema, and cannot, I think, be regarded as wholly attributable to it. On the other hand, there is a suggestive connection between the occurrence of this œdema in the neighbourhood of the operative tissue-injury, the increased permeability of the capillaries in general, and the extreme sensitiveness to histamine which follows the operation. It would seem that the animal, through loss of its suprarenal glands, has already been brought almost to the limit of its resistance to the toxæmia following the inevitable trauma, so that a normally trivial dose of histamine suffices to turn the scale in the direction of fatal collapse.

DISCUSSION.

The practical bearing of these observations is evident. Evidence obtained during the war had pointed to an aseptic traumatic toxæmia, due to absorbtion of substances produced by injury and autolysis of tissues, as a central factor in the condition termed "secondary wound-shock." There were, however, various accessory and complicating factors—exhaustion, pain, cold, thirst, hæmorrhage, anæsthetics—the precise *rôle* of which was always difficult to estimate. I have examined the effect of certain of these factors, which were suitable for laboratory experiment, and found that they lower in a very striking degree the normal resistance to histamine. It seems probable that the resistance of the otherwise perfectly healthy subject to products of tissue injury would similarly be high, and it is reasonable to attribute the demonstrated danger of chloroform or ether to the severely wounded man, and the relative safety of nitrous oxide, to the much greater effect of the former anæsthetics, as shown in these experiments, in depressing the normal resistance to this type of toxæmia. It seems possible, further, that the greater safety in civilian operations, as shown by Crile and others, of the combination of local anæsthesia with nitrous oxide and oxygen, as compared with prolonged anæsthesia with ether or chloroform, may find its true explanation along the same lines. Certainly, I think, the conclusion is warranted, that in cases in which bacterial or other toxæmia is present chloroform and ether should be avoided whenever possible.

The weakening of resistance by hæmorrhage needs little comment. The necessity of restricting the loss of blood in operation, if shock is to be avoided, has long been recognised. I think it is justifiable to suppose that the significance of the hæmorrhage, like that of the anæsthetics, lies not so much in the harm which it would do by itself, as in its effect of lowering the threshold of tolerance to poisonous products from injured tissues.

The effects of removing the suprarenal glands are at once the most striking, the most suggestive, and the most difficult to interpret. We are dealing with a complex of effects produced by removal of both the cortex and the medulla of the glands. The present trend of opinion seems to be in favour of regarding the fatal effect of the removal as due to lack of the cortex. If we are to regard the extreme sensitiveness to histamine as due to the absence of this part of the gland, the observation can simply be taken at its own value. We can only note the probability that poisonous substances of the histamine type are probably at all times being formed in the body in small quantities; the resistance of the normal animal to their action may, therefore, give a hint of an unknown function of the suprarenal cortex, and of the cause of the death which rapidly follows its ablation. On the other hand, there are points of support for the view that the secretion of adrenaline by the medulla of the gland is, at least, an important factor in the normal resistance to histamine. It is easy to demonstrate that a small dose of histamine accelerates the secretion of adrenaline in the normal animal. In a cat from which one superior cervical ganglion has been removed some days previously, the pupil on the side of the operation has a greatly accentuated sensitiveness to adrenaline. If 0·01 to 0·1 mgrm. of histamine is injected into the ear-vein of such a cat a pronounced dilatation of the sensitised pupil results. The effect is exactly that of injecting a small dose of adrenaline, or of any influence causing a small acceleration of its secretion from the suprarenal glands.

If the records given above, of the results of infusing large doses of histamine into normal cats, be consulted, it will be seen that wide dilatation of the pupils accompanies the infusion. My experimental records seem to show that when, as in Cat 1, this dilatation is pronounced and persistent, the symptoms produced by the infusion are relatively slight and evanescent, but that when the dilatation is not well marked, and passes off in the later stages of the infusion, the symptoms are more severe and recovery is delayed. Cat 11, for example, a week before the suprarenal glands were removed, received the usual test-infusion of 10 mgrm. of histamine per kilo. My note records that the pupils throughout the infusion showed little dilatation, and that when half the dose had been administered they were not dilated at all. At the end of the infusion the cat was deeply narcotised, and needed some hours for complete recovery. It must be admitted that such observations are suggestive rather than convincing. If we had clear evidence that the extreme sensitiveness to histamine, caused by extirpation of the suprarenal glands, was due to lack of adrenaline, it would be justifiable to consider whether this lack were not the common factor in all the conditions by which such sensitiveness has been found experimentally to be enhanced—anæsthesia with ether, hæmorrhage, adrenalectomy—or which clinical observation has recognised as contributory to the production of shock—exhaustion, excitement, pain. I do not think, however, that the evidence yet available is sufficient to bear this weight of interpretation.

SUMMARY.

Histamine can be given by intravenous infusion to a normal cat, not under an anæsthetic, in doses up to at least 10 mgrm. per kilo., without severe or lasting effect.

In the same animal, at the end of anæsthesia for an hour or more with ether, 1 to 2 mgrm. of histamine per kilo. will produce the characteristic fatal "shock." Anæsthesia with nitrous oxide and oxygen has not this effect of lowering the resistance to histamine.

A large but not in itself serious hæmorrhage greatly reduces the resistance to histamine.

On the day following operative removal of the suprarenal glands the resistance of cats to histamine was found to be reduced to a very low level, as little as 0·16 mgrm. per kilo., given without anæsthetic, having proved fatal in one instance.

The circulatory condition developing in the twenty-four hours following removal of the suprarenal glands has features in common with that produced by histamine, including a pronounced increase in the proportion of corpuscles in the blood.

The bearing of these observations on the nature of the conditions conducive to the production of traumatic shock is discussed.

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THE COMPARATIVE OXYGEN AVIDITY OF NORMAL AND MALIGNANT CELLS MEASURED BY THEIR REDUCING POWERS ON METHYLENE BLUE.

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It has been known since the work of Ehrlich (1) that fresh tissues and, in some circumstances, extracts of tissues possess the power of decolourising methylene blue in the presence of water. Schardinger (2) found that fresh milk would rapidly bleach solutions of methylene blue, provided that an aldehyde such as acetaldehyde or formaldehyde was present, and that this property was destroyed by boiling. Bach (3, 4) showed that an extract could be prepared from such tissues as liver and kidney which would reduce methylene blue in the presence of acetaldehyde, and this enzyme apparently reduced nitrates to nitrites also.

Some time ago it occurred to me that a measure of the relative oxygen avidities of normal and malignant cells might be obtained by observing the amount of methylene blue which such cells would reduce. It has been found that both normal and tumour cells will live for some time in Locke's solution, and this fluid, omitting the glucose, was therefore used as the diluent throughout these experiments. At first varying concentrations of methylene blue were tried, varying from 0·2 per cent. to 0·001 per cent. Of these the most suitable strengths were found to be either a ·001 per cent. or a ·002 per cent. solution of the dye in Locke's fluid. I have found it convenient to use special test-tubes for these experiments, each tube being 20 cm. long by 0·5 cm., the capacity being 10 c.c. The tissues are removed with aseptic precautions, and are minced either with scissors or with the mincer designed by Haaland. Weighed or measured quantities (0·3–0·5 c.c.) of the tissues to be studied are placed in these tubes by means of a glass syringe and long hollow needle, and the tubes filled up with a measured amount of the methylene blue solution. A layer of paraffin is poured on to the surface to prevent re-oxidation of the methylene blue, and the tubes are then incubated at 37° C. The effect noted is a progressive decolourisation of the blue fluid, beginning at the bottom next the tissue, and gradually extending upwards. Readings are made either every half-hour, or hour, up to five or six hours. A reading consists of measuring with a millimetre rule the height of the column of methylene blue decolourised by the tissues. This may be quickly and accurately done by means of a special rack designed for these tubes.

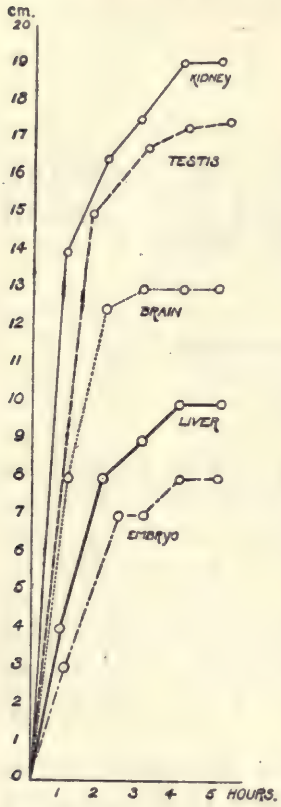

Fig. 1.—Curves of decolourisation of methylene blue by normal tissues.

A great variety of normal mouse tissues was first examined in the manner described, including kidney, liver, spleen, testis, heart, brain, mammary gland, connective tissue and embryo. The height of the column of methylene blue reduced, though varying, is very similar for the normal tissues, brain and spleen giving the lowest readings, and kidney and testis the highest. In order to compare the results from different experiments the heights of the columns of decolourisation were plotted as ordinates against the time as abscissæ. When this is done all the normal tissues give curves of the same general character (Fig. 1). The curve rises steeply from the start, and gradually slows down in the second, third and fourth hours, after which only minimal quantities of dye are bleached.

Having examined many normal tissues in the manner described, a great number of times, I next examined a number of transplantable mouse carcinomata (Jensen, Twort, 63, 113, 206), a mouse sarcoma (37 S.), a rat sarcoma (Jensen), and a scirrhus mammary carcinoma from the human subject. A very striking difference became apparent with all these tumours, when compared with normal tissues. Thus, taking kidney as an example of a normal tissue, we find, on examining the curve produced, a great rise in the first hour, followed by smaller but continuous rises up to the fourth or fifth hours, when reduction usually ceases. In a tumour, however, such as Jensen's adenocarcinoma, the curve is quite different. No apparent reduction takes place for the first hour or two. A small rise then takes place, ceasing, usually, about the fourth or fifth hour, when transplantation experiments show

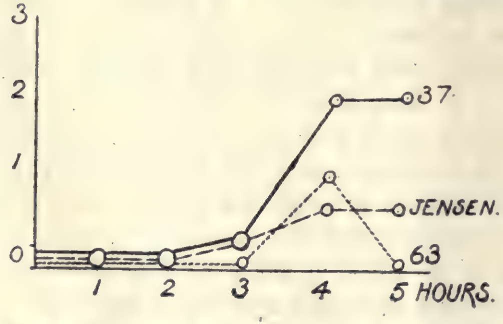

Fig. 2.—Curves of decolourisation of methylene blue by mouse tumours.

the cells to be dead, and microscopical examination shows that the mitochondria have become either very degenerate or have disappeared altogether. Fig. 2 shows curves obtained from Jensen's adenocarcinoma, 37 S. and 63, whilst Fig. 3 shows a curve from 113. All these tumour curves appear to be very similar, the only one showing a striking difference being 113. This tumour is particularly interesting as it contains considerable amounts of glycogen. Amongst all these tumours, leaving aside the exceptional case of 113, those showing the greatest reducing powers are 37 S. and 63, and this is interesting as both are of rapid growth.

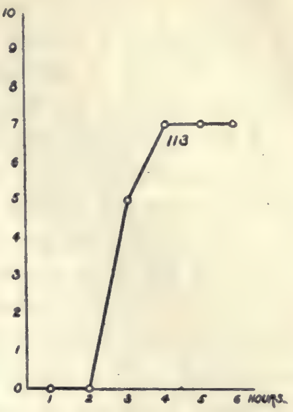

Fig. 3.—Curve of decolourisation of methylene blue by a glycogen-containing mouse tumour (strain 113). Apart from the initial lag, this tumour approaches closely to the behaviour of normal tissues.

Cramer (5) showed some little time ago that if tumour cells were shaken with a soluble ionisable calcium salt a profound depression was produced, and the percentage of "takes" on transplantation fell considerably. Tumours exhibiting this artificial depression could, however, be restored by shaking with sodium salts. This depressant action of calcium salts is very well shown by testing tissues, such as kidney, with methylene blue, after shaking with a soluble calcium salt such as the nitrate. Fig. 4 illustrates this action well. The curve is taken from kidney, which had been shaken for fifteen minutes with a M/7 solution of calcium nitrate. It will be noticed that the resulting curve is very similar to those obtained with tumours, there being the same initial delay. Fig. 4 also illustrates the restorative action of sodium salts, the kidney being shaken with M/7 sodium chloride, after the treatment with calcium. The curve obtained is a normal one.

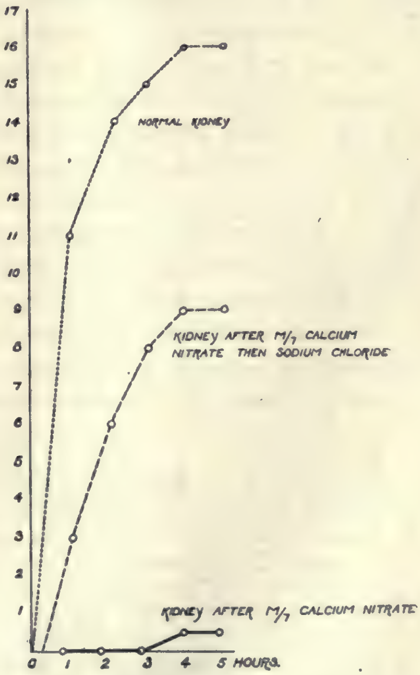

Fig. 4.—Depressant action of calcium salts on the reducing power of kidney, and restoration of this power by washing out the calcium with sodium chloride solution.

That the living cell is necessary for this reaction is shown not only by the effect of calcium salts, but also by the fact that if the tissues be frozen and ground up till no intact cells can be demonstrated by the microscope, very little reduction takes place. This may be conveniently shown with kidney by finely mincing the organ and then grinding up in a mortar which is placed in a freezing-mixture. The necessary amount is then placed in one of the tubes, filled with Locke's fluid, and centrifuged for a minute to carry the fine particles down, the supernatant fluid is pipetted off and the methylene blue solution added.

If a dilute solution of hæmoglobin be made in Locke's fluid the reduction may be read off with a direct vision spectroscope and recorded in the same manner as with the methylene blue, and if this be done it is seen that the curves obtained are quite similar to those obtained by means of the methylene blue reaction. Further investigation showed that the apparent lag exhibited by all tumours can be abolished if the methylene blue solution be thoroughly exhausted at the pump and washed with pure hydrogen three or four times, exhaustion being carried out till the solution boils between each hydrogen wash. Normal tissues placed in such hydrogen-washed fluids reduce the blue at a very rapid rate and to a greatly increased amount, whilst tumours, although reducing much less than the normal tissues and not nearly so rapidly, show no lag as measured by the hourly readings.

It would appear to be reasonable to conclude that the reduction of the methylene blue is a measure of the oxygen taken up by the cell as the process appears to follow the hæmoglobin curve. Methylene blue, however, contains no oxygen, and the reduction to the leuco-base is entirely due to the addition of nascent hydrogen atoms to the molecule, the hydrogen apparently being obtained from the intracellular water whilst the oxygen is utilised by the cells. The lag which is noticeable in the tumours is probably due to the fact that they have a low oxygen avidity and that the slight amount of oxygen dissolved in the solution is enough for their needs for some time. If, however, the solution be exhausted and washed with an indifferent gas, then, there being no free oxygen to hand, the cells have to obtain it from the solution and consequently reduce the methylene blue.

Further investigations, now in progress, will be required to determine whether the reaction can be used to distinguish neoplastic from other pathological proliferations, or benign from malignant tumours.

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CRITICAL REVIEW.

THE ÆTIOLOGY OF INFLUENZA.

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THE recent epidemic of influenza witnessed a great revolt against the view, up to then generally accepted, that the organism, *B. influenzæ*, discovered by Pfeiffer in 1892, was the cause of the disease.

An opposition to the Pfeiffer discovery had, indeed, developed very soon after its publication and had been maintained intermittently to the time of the recent epidemic. This opposition was based upon the fact that *B. influenzæ* was so often absent from small outbreaks of influenza which occurred after the discovery of the bacillus, while, on the other hand, it was found in large numbers in other outbreaks. Furthermore, the bacillus began to be described in other diseases which had no connection with influenza and even in normal persons. Thus it was not clear upon what grounds it should be considered the cause of influenza any more than the streptococci or pneumococci which were frequently found in this disease and were equally wide-spread.

In spite of these objections, the majority of bacteriologists supported the view that *B. influenzæ* is the cause of influenza, although it may be admitted that a considerable proportion had little or no experience of this organism in its relation to the epidemic disease.

When the recent pandemic began to assume serious proportions bacteriologists set themselves to find *B. influenzæ* with the methods described by Pfeiffer and it is now common knowledge that they largely failed. It is thus not surprising that the anti-Pfeiffer school received many adherents.

Very soon the cause of influenza began to be ascribed to certain invisible or almost invisible bodies, and at one time these "filter-passers" were widely regarded with favour.

Meanwhile the arguments of the more cautious bacteriologists that *B. influenzæ* was after all nearly always demonstrable in influenza if proper methods were used, received scant attention. Since Pfeiffer, in 1892, had laid such stress upon the enormous numbers of bacilli present and the purity and copiousness of his growths, it was thought idle to plead that success depended upon the use of some newly-discovered culture medium.

Following the epidemic a large amount of work was carried out upon the ætiology of influenza, without, however, reaching a conclusion. This work showed that the Pfeiffer school was far from being disposed of; in fact an increasing mass of material was collected which favoured *B. influenzæ*, while, on the other hand, little advance was made in the filter-passing concept of the disease.

It may be remarked that much bacteriological work upon the ætiology of disease suffers from the drawing of speculative conclusions and a disregard of logic in deducing the value of experimental results. Thus in making an attempt to review the claims of *B. influenzæ* on the one hand and filterable viruses on the other as the immediate cause of influenza, it will be necessary to notice shortly the fundamental grounds upon which the causation of a disease may be ascribed to a presumed virus. These grounds are summarised in the so-called "postulates of Koch," which are not, however, to be accepted as hard and fast rules. They must be construed in the light of the knowledge of disease as it exists at the time being, but modifications should be made with the greatest caution. It may be said to be certain that the number of mistakes which have been made is in direct proportion to the amount of deviation from Koch's principles.

THE EVIDENCE UPON WHICH A VIRUS MAY BE ASSOCIATED WITH THE CAUSATION OF AN INFECTIVE DISEASE.

So far as we have been able to discover, Koch did not tabulate any definite "laws" such as are often quoted at the present day.

Prof. William Bulloch has called our attention to Friedrich Loeffler's 'Vorlesungen über die geschichtliche Entwickelung der Lehre von den Bakterien.' (1) Loeffler, indeed, refers to "postulates" . . . "Constanter Nachweis, Isolirung und Prüfung der isolirten Organismen, . . . " but these are not Koch's postulates, but Henle's, formulated thus crisply by Loeffler. Henle, in his 'Pathologische Untersuchungen,' (2), says: "If one finds living motile animalcules . . . in the contagious material, it is possible . . . that they are there accidentally, if the material has been exposed for some time to the air. Even if they are found constantly in the contagious material and inside the body, it is possible . . . that they are merely parasitic elements, which develop in the fluid and may be of diagnostic importance, without being the active principle, as some still say of the spermatozoa in the semen. The only way to decide whether one is the active principle . . . is to isolate the spermatozoa, seminal fluid, contagious organism and contagious fluid and test the power of each . . ."

This is an early and interesting deduction from purely theoretical considerations, and, indeed, contains the nucleus upon which Koch worked.

In 1878 Koch (3) stated in italics that the parasitic nature of a disease can only be certain when "the parasitic micro-organisms are to be found in all cases of the disease in question, when, further, they are demonstrable in such numbers and distribution that all symptoms of the disease are thus explicable by them, and, finally, when a morphologically well-characterised micro-organism is established as a parasite in every individual traumatic infective disease."

In 1884, having in the meantime discovered the method for the pure cultivation of bacteria, he is more explicit (4). To establish that a bacterium is the cause of tuberculosis, it is necessary, "first, to determine whether formed elements, which could neither belong to the constituents of the body nor be derived from them, are present in the lesions. . . . Whether they show any sign of possessing independent life . . . It must be shown that the parasites directly produce the disease. To obtain this proof, it is necessary to isolate the parasites . . . then to produce the disease anew with all its characteristic features by introducing the parasites alone into a healthy organism."

These are the clear principles upon which Koch worked and which are now referred to as his "postulates."

Thus to be established as the cause of an infective disease, a virus should fulfil all of three main conditions:

(1) *The virus should be capable of being recognised in a large proportion of the cases of the disease, preferably in relation to the chief lesions.*

In practice it is obviously idle to demand that the virus shall be found in every instance of a disease. The varying skill of the investigator, serious technical difficulties or, again, lack of time or facilities may make it impossible to demonstrate a special character, such as the presence of a virus, in all of a large series of cases.

Further, when the relation of a virus to a disease is under consideration, it must, of course, be understood that a "disease" in common parlance is a clinical syndrome more or less accurately recognised, and that such a syndrome is not necessarily due to a single cause. It may be recalled that the term "general paralysis" is still applied to conditions which are not syphilitic, and therefore it cannot be expected that the syphilitic virus will be found in all cases of "general paralysis" diagnosed by persons who are not familiar with the modern ætiological classification of diseases. It is only with increasing knowledge due to the close study of cases of proved ætiological similarity that those signs or symptoms are at length recognised, which indicate clinically the type of disease in which the specific virus should be demonstrable.

It is more convincing evidence of cause and effect if a virus is found alone in intimate relation to a chief or characteristic lesion of a disease; thus the meningococcus in the cerebrospinal fluid is of more obvious ætiological importance than even the pneumococcus in the pneumonic lung, the latter being often associated with large numbers of other bacteria. Koch held that bacteria should be present "in such numbers and distribution that all the symptoms of the disease may be thus explained," but it should be understood that "such numbers" does not necessarily imply large numbers, nor "distribution" a concentration in the lesions. The chief characteristic lesion of a disease may be due to toxins emanating from a small quantity of a virus situated at a distance, viz. the nervous lesions of diphtheria or tetanus.

The demonstration of the virus may be (*a*) by reproduction of the disease in other animals, or (*b*) by visible demonstration or cultivation.

(*a*) This method of demonstration is particularly applicable to viruses which are "invisible" or have not been seen. Thus the syphilis virus, before the discovery of *S. pallida*, was shown to be present in chancres by the reproduction of these lesions in animals with material taken from chancres, or the virus of typhus is known to be present in the blood of patients owing to the infective action of this blood upon guinea-pigs.

(*b*) By staining or by other methods the virus should be demonstrated as an abnormal structure of characteristic appearance in the lesions of the disease.

The appearance of such a structure may be so abnormal and may bear such a relation to certain features of a disease that its mere demonstration has been accepted as indicating the cause of a disease. Thus *B. lepræ* has not been certainly cultivated nor has it been conclusively proved capable of transmitting leprosy, but it has nevertheless been accepted as the cause of this disease.

Some viruses are present in lesions in such small quantities that a visible demonstration is impracticable; in such cases—for instance, in sporothricosis—an abnormal structure may be grown and the disease ascribed to it with some degree of probability.

If a virus can be demonstrated to be present in the lesions of a disease with relative frequency, its ætiological importance may be accepted provisionally in spite of the fact that it may also be present in other parts of the body where no obvious lesion exists, or indeed, in spite of the fact that it may often be found in association with other diseases or in healthy persons. The idea that a virus occasions disease whenever introduced into the body is no longer tenable. The development of a disease depends upon two factors, namely, the introduction of the virus in a suitable form or under suitable conditions, and the susceptibility of the individual; thus pneumococci, streptococci, meningococci and other bacteria may be present in many persons who are healthy, and yet this fact does not detract from the ætiological importance of these bacteria in the diseases ascribed to them. We may further instance the fact that

Amœba histolytica is accepted as the cause of amœbic dysentery although they are commonly found in the stools of healthy persons who have never had dysentery.

On the other hand, a pathogenic virus may be found in diseases other than that with which it is chiefly associated. For instance, pneumococci are frequently present in the lungs in phthisis although they are not the cause of the disease. They are "secondary infectors," but this does not weaken their claim to be the prime cause of lobar pneumonia.

The ascribing of a disease to a virus merely upon the optical or cultural demonstration of its presence in the lesions is, of course, always dangerous for the reason, already noted, that many viruses are commonly present in the healthy body as "saprophytes," and are thus available to take part acutely or mildly in a secondary infection. No doubt in some cases the deduction has been correct, but in others very questionable—for instance, the relation of streptococci to rheumatic fever. The history of the discovery of the causation of disease is strewn with errors due to these hasty conclusions, a recent example being the description of *B. suipestifer* as the cause of hog-cholera—a condition now known to be due to an invisible virus.

(2) *The virus should be shown to be living.*

The causative virus of an infective disease must be proved to be living and not merely a poisonous or irritant substance incapable of propagation in series. Thus, while various pollens may be accepted as the virus of hay-fever, this disease is not infective and the virus has no power of direct spread to a healthy individual. It is no more an infective agent than egg-white is infective when it happens to be swallowed by a susceptible person.

The characteristic of life, meaning the power of indefinite propagation under suitable conditions, may be demonstrated in two ways:

(1) By passage of the virus through a series of animals.

(2) By a similar passage through a series of generations in artificial culture.

The success of these methods of demonstration depends, of course, upon the discovery of a suitable animal or a suitable medium in which the virus will grow. We have already referred to the fact that the virus of typhus will propagate itself through a series of guinea-pigs, while rabies has been carried for years through innumerable rabbits in this manner. On the other hand, actinomycosis has not yet been proved to be a living virus by passage through animals because, although it is known to be pathogenic to bovines, the precise method for successful transmission has not yet been discovered.

Similarly, with artificial cultures many viruses may be propagated *ad infinitum*, but in other cases the essential medium or other condition for success is not yet available.

The determination of the fact of culture in series is not always easy. Twort (5), for instance, described a property which he had found in vaccine lymph which had the effect of killing and dissolving certain cocci which he had isolated from the lymph. This property was capable of being carried indefinitely in series through suspensions of the cocci and thus gave the impression of being a living virus capable of growth. There is, however, more reason for supposing that the property is an autolytic ferment derived from the cocci themselves. When minute quantities are inoculated into a suspension of the cocci, these are dissolved with the liberation of more ferment; this "culture," upon being transferred, repeats the process, thus simulating growth and life. Twort's phenomenon, upon being rediscovered by d'Herrelle (6), was ascribed to a filter-passing organism—"microbe bactériophage."

(3) *The virus should be capable of reproducing the disease in other animals.*

The final criterion by which a virus may be adjudged to be the cause of a disease is its power of reproducing this disease when introduced intentionally into a healthy animal.

The inoculation should, of course, be carried out with a *pure* culture of the virus, but if it is not capable of being cultivated other methods must be found to ensure that the virus inoculated is a *pure* virus and not contaminated with some other which has been overlooked. Thus the association of malaria parasites with malaria was proved by the purification of the virus by passage through the mosquito before it was inoculated into man.

Further, it is not enough to show by inoculation that a pure virus is capable of producing a disease; it must be shown by passage that the disease produced is itself infective and due to a living virus and not to the irritant action of its constituents. It has, for instance, been claimed that a gonorrhœal infection can be produced by inoculating gonococci into the anterior chamber of the eye but, as Mezincescu and Holban (7) pointed out, this is not an infection, because the condition cannot be reproduced by transferring the pus into the eye of another rabbit. No gonococci are demonstrable in the discharge and, further, a similar lesion can be produced with dead gonococci. Thus the ophthalmitis is not a gonococcal infection in the rabbit but a non-infective lesion due to an inanimate poison.

Great difficulties sometimes arise in establishing the fact of a successful transmission of a disease. Thus, it is not to be expected that all diseases in man will be capable of being typically reproduced in animals. Obviously the outstanding symptoms of a disease in man may be due to anatomical features which animals do not possess, or may be due to methods or routes of infection to which animals are not susceptible or are not exposed. A virus supposed to be the cause of a particular illness in man may be introduced into an animal and may cause disease or death, but the essential matter to determine is whether this disease is the same disease as that which it is supposed to have caused in man.

This is a question which can seldom be settled by noting the clinical symptoms, because animals are little adapted to this method of examination. It must be settled by pathological methods.

Now, the gross pathological features of a disease are very largely governed by the method of infection and the susceptibility of the individual. Bubonic plague following infection through the skin of the leg has little in common with pneumonic plague derived from the air-passages. Similarly typhoid in the rabbit is quite different according as the virus is introduced into the peritoneal cavity or, suitably, into the stomach. The latter route reproduces the features of typhoid in man (Besredka (8)), the former does not—a different syndrome is produced.

The virus of syphilis in man produces a generalised disease, but in the rabbit the lesion is practically confined to the point of inoculation owing to the different susceptibility of this animal.

In general it may be supposed that the virus from every human infective disease *could* reproduce a closely similar disease in animals if it were always known *how* to introduce it or into what *species* of animal to introduce it. If the conditions were known under which the meningococcus, for instance, established an infection in man, it might be possible to produce meningitis in animals. Since this is not yet known meningitis cannot be produced, although peritonitis can be demonstrated in young guinea-pigs.

Thus in many cases the experimenter must perforce be content to produce in animals, not the characteristic disease of man, but another characteristic disease of animals, and he has to determine by microscopic methods or by analogy or by other methods whether this disease which he produces is comparable to the natural disease in man—in other words, whether the virus, supposed to be the cause of a disease in man, has in fact reproduced it in animals.

The method of demonstrating the existence of immune reactions in infected animals which are similar to immune reactions found in cases of human disease is often used to suggest the presence in both cases of the same virus. This is quite legitimate, but it is certainly not legitimate to assume that the virus which is present in both is the cause of the same fundamental disease in both. For instance, a *proteus* bacillus may be isolated from cases of typhus, and upon injection into an animal may produce agglutinins which are similar to agglutinins detectable in the blood of a typhus patient. From this it may be deduced that the case of typhus is infected by the *proteus* bacillus, but not that this bacillus is the cause of typhus. This disease is known to be due to another virus. The discovery of these immune reactions merely suggests an infection, which may be secondary, and cannot be accepted as proof of a successful transmission of a disease.

Having thus considered the modern application of Koch's criteria, their relation to research upon the ætiology of influenza will now be reviewed.

(*To be concluded.*)

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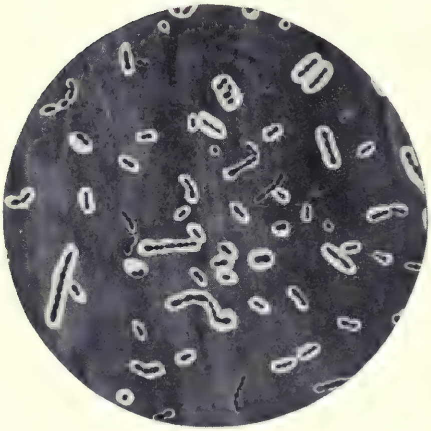

FIG. 1.—*Streptococcus mucosus.*

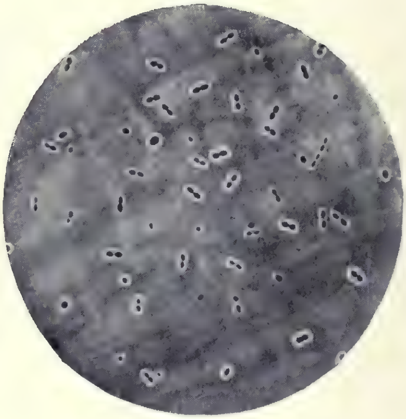

FIG. 2.—Pneumococcus.

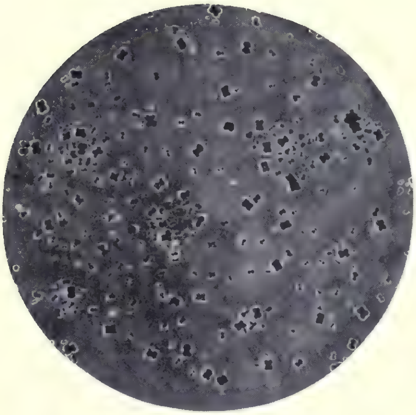

FIG. 3.—Meningococcus.

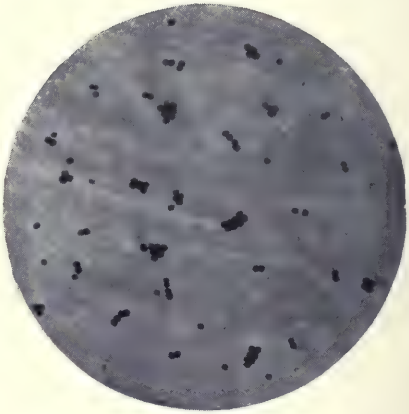

FIG. 4.—*Staphylococcus aureus.*

Baker. Capsules of Bacteria.

TECHNIQUE.

TECHNIQUE FOR THE DEMONSTRATION OF THE CAPSULES OF BACTERIA.

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Received for publication January 31st, 1920.

THE following is a simple and rapid method for examining an organism for the presence of capsules.

It is applicable to organisms in culture or in cerebro-spinal fluid or sometimes in thin pleural exudates, but cannot be used satisfactorily when much pus or blood is present.

METHOD :

Make a film of the organism as for ordinary staining.
Dry by moderate heat.
Spread a film of Indian ink upon the dried film. Higgins' waterproof Indian ink is recommended.
Dry the ink in the hot air over a bunsen burner.
Stain 1 to 2 minutes in hot gentian violet or carbol-gentian violet.
Wash off the superfluous stain under the tap and add Gram's iodine for a minute.
Blot dry.

The above procedure gives an intensely black organism in the middle of a perfectly transparent capsule and makes it possible to detect the slighter degrees of capsule development.

The following points should be noted :

(1) A good homogeneous background of ink is necessary : this is impossible in pus and is often interfered with by other fluids such as broth, so that the best results are obtained by emulsifying in water a loopful of a slope culture.

(2) Alcohol fixation of the film before spreading the ink gives poor results as it leads to deposition of the ink on the capsules.

(3) The staining should be as intense as possible, since if the organism is not perfectly opaque to light its transparent margin may give the false impression of a capsule. This is seen in any dead and poorly staining

organisms which may be present; they appear as white spaces or more often as slightly coloured spots with white margins.

(4) On keeping, the black iodine-treated specimens fade to a violet, which, however, still shows the capsule satisfactorily as a rule; if necessary they can be washed in xylol and alcohol and re-stained without damage to the ink film.

The plate shows four organisms with different degrees of capsule development.

The pneumococcus is not satisfactorily dealt with by the above technique since its capsule is relatively easily stained and therefore does not show up as a white area: in this case the specimen should be washed in alcohol after the iodine as in Gram's stain.

The staphylococcus shows no appreciable capsule by this method. Occasionally a specimen is found with a barely visible bright line at the edge, but this is on one side only and appears to be due to slight contraction or shifting of the organism.

It is generally recognised that Indian ink gives the correct outline of organisms and this applies to the capsular outline, but by the use of too thick a film the organism may be partly covered up and the capsule obscured.

Shrinkage during the staining process is inappreciable; there may be slight shrinkage or displacement of a few of the organisms but this is insufficient to give the effect of a capsule.

A NEW MEDIUM FOR THE GROWTH OF *B. INFLUENZÆ.*

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(*A report to the Medical Research Committee.*)

Received for publication February 5th, 1920.

WHEN Matthews introduced his trypsin-digested medium for the growth of *B. influenzæ*, I found it inconvenient to use human blood and had recourse to ox and sheep's blood. Trypsin, however, does not digest these bloods to the same extent as human; even when the digest has been maintained at 37° C. for a number of days, there is always a precipitate of proteins when it is mixed with agar.

The effect of peptic digestion was then tried, and it was found that such a digest even after only 30 minutes at 55° C. could be rendered faintly alkaline and mixed with agar without any precipitate occurring.

This peptic digest grows *B. influenzæ* in the same copious manner as does Matthews' medium, and it has the advantage of being much more rapidly prepared and more constant in action. Sterility of the initial reagents is of no consequence. Samples of the digest have been kept at room temperature for over twelve months without undergoing deterioration.

The following is the manner of preparation:

Into a 250 c.c. bottle, fitted with a well-ground glass stopper, place in the indicated order—

(1) 150 c.c. saline solution, not distilled water.
(2) 6 c.c. pure HCl.
(3) 50 c.c. defibrinated sheep's blood.
(4) 1 grm. pepsin, B.P., granulated.

Shake to dissolve. Place in the water bath at 55° C. for 2 to 24 hours, shaking occasionally at first. The exact time is unimportant. Then add 12 c.c. of 20 per cent. NaOH, accurately prepared, and test the reaction with cresol red (·02 per cent. solution). If this dye does not give a colour of permanganate, continue adding 20 per cent. NaOH until this point is reached (P_H 7·6). For the purpose of testing, the samples are removed from the bottle with a sterile pipette and diluted with distilled water until the colour is light enough to show the change on the addition of a few drops of cresol red. When the correct point is reached add pure HCl drop by drop until cresol red gives practically no change of colour, but phenol red gives red (P_H 7·0 to 7·2). Finally add 0·25 per cent. of chloroform, slightly grease the stopper and shake to dissolve the chloroform. Protect the bottle from dust with a paper cap.

For use, shake the bottle, remove the material with a sterile pipette and add it directly to melted agar in a strength of 2 to 5 per cent. or to broth in the same proportions. It is not necessary to remove the chloroform.

The correct adjustment of the reaction is essential; if the bottle is left too alkaline the contents will rapidly deteriorate. On standing a slight precipitate of hæmatin takes place and the bottle should be shaken before use, but if the reaction is too acid the precipitate will be excessive and the quality of the medium will suffer accordingly.

THE BRITISH JOURNAL

OF

EXPERIMENTAL PATHOLOGY

THE ANTIGENIC PROPERTIES OF *B. PSITTACOSIS.*

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THE following investigation was undertaken to establish the identity of an organism isolated from a bird which sickened and died from an illness in which enteritis was a prominent symptom.

This bird, a grey touracou (*Schizorhis concolor*), belonging to a species native to South Africa, is entirely a fruit eater. It, together with six others of the same species, was imported into this country in December. A few weeks after arrival one died, and three others contracted, at short intervals, a fatal illness characterised by enteritis. Two only of the batch have survived. Post mortem, the intestines of the fatal cases were found to be congested and inflamed, and in one a well-marked peritonitis was present.

The bacillus, which was isolated from the heart-blood, spleen and intestinal contents, conformed in its morphology, staining and biochemical reactions to a member of the Salmonella group of organisms. It was a short bacillus with rounded ends, and was very actively motile. It was Gram negative. Cultures were readily obtainable on all media. It did not ferment lactose; glucose, mannite, and dulcite were fermented with the production of acid and gas. Indol was not formed.

Its pathogenicity was tested and it was found virulent for the following animals:

Mice.—Subcutaneous inoculation of 0·2 c.c. of a twenty-four-hour broth culture caused a septicæmia which proved fatal in 12 hours. The organism was recovered from the heart-blood, spleen, urine and intestinal contents.

Rabbits.—Intravenous inoculation of 0·3 c.c. of the same culture killed rabbits of 760 grm. weight in 48 hours. Positive cultures were obtainable from the heart-blood, spleen, bile, urine and intestinal contents.

Pigeons.—Feeding experiments were not successful. Intra-peritoneal inoculation of 0·2 c.c. killed a bird in 8 hours. The bacillus was recovered from the heart-blood.

The organism, considering its cultural reactions and source of origin, could be classified with the bacillus of psittacosis.

A short summary of the earlier work connected with this organism is of interest. The first human epidemic that can be attributed to it was described by Dujardin-Beaumetz (1) in 1892. A fatal form of infectious disease made its appearance amongst a consignment of parrots during transit to Paris from abroad. Three hundred birds died out of a total of 500. The surviving birds were distributed to various centres and human contact cases originated in the neighbourhood of many of them. In all 42 cases were reported in which the illness could be traced to this source of infection, and fourteen deaths occurred during the epidemic.

Dujardin-Beaumetz at first considered the disease to be of the nature of an infectious influenzal pneumonia, and whilst forming the opinion that it could have been spread from man to man through the intermediary of the parrots, he did not think it was a specific infection of these birds.

The following year Nocard (2) isolated an organism which he believed was the causal agent of this outbreak. As he did not have at his disposal any affected parrots he was obliged to limit his researches to the bone-marrow obtained from the wings of some of the birds that had died. He succeeded in isolating an organism, which he found not only pathogenic for parrots, but which also possessed a high degree of virulence for all laboratory animals.

Up to this time the organism had not been recovered from a human case of psittacosis, but this was accomplished later by Gilbert and Fournier (3) during the course of another small outbreak. They were able to isolate, both from the patients and from the organs of the diseased birds, a bacillus which corresponded with that described by Nocard.

B. psittacosis has, in view of its biochemical and cultural characteristics, been included in the Salmonella group of organisms. This group, including as its principal members *B. enteritidis* (Gaertner), *B. paratyphosus B*, and *B. aertrycke* (*suipestifer*), was rendered more complex by the admission of a number of other organisms obtained from various sources other than human, the more important of these being *B. typhi murium*, *B. danysz*, and *B. psitta-*

cosis. The work of Bainbridge (4) and others identified these latter bacilli with one or other of the principal members of the group, *B. psittacosis* being apparently identical with the strain of *B. aertrycke* employed in the comparison. However, it has recently been shown that considerable variation in antigenic properties exists between different strains of *B. aertrycke*, and that the serological differences brought into evidence by the absorption test are sufficient grounds for the recognition of various types of this organism. Schütze (5) (6), studying this question in detail, has recognised eight different types, the most common being a strain isolated from cases of food poisoning which is known as the "Mutton" type. According to this observer over 50 per cent. of "Aertrycke" bacilli, including organisms isolated from animal and human sources, are identical in antigenic properties with this strain. All of these are believed to be capable of causing an acute gastro-enteritis in man.

The object of this investigation was to determine (1) if this bacillus, definitely of avian origin, could be identified by its antigenic properties with one of the recognised types of *B. aertrycke*, and (2) what relationship it bore to the organism originally isolated by Nocard.

An agglutinating serum for the bacillus was prepared and its titre determined for the homologous organism *B. paratyphosus B*, and two types of *B. aertrycke*—"Mutton" and "Newport." Absorption tests were then made with each of these organisms, absorption with the homologous bacillus serving as a control of the technique employed. The results are given in the appended table.

Serum.	End point of agglutination titre for:			
	Bacillus "X"	*B. aertrycke* ("Mutton")	*B. aertrycke* ("Newport")	*B. paratyphosus B.*
Bacillus "X"*	25,000	25,000	4000	4000
Ditto absorbed with—				
1. Bacillus "X"	< 500	< 500	< 500	< 500
2. *B. aertrycke* ("Mutton")	< 500	< 500	< 500	< 500
3. *B. aertrycke* ("Newport")	20,000	20,000	< 500	< 500
4. *B. paratyphosus B*	20,000	20,000	< 500	< 500

* The organism isolated from the bird is referred to in the above table as Bacillus "X."

It will be noted, on reference to these experiments, that the absorptive capacity of the Aertrycke "Mutton" strain corresponded with that of the organism under investigation. Both the specific and group agglutinins were removed from a serum prepared with this bacillus. Saturation with the Aertrycke "Newport" strain and *B. paratyphosus* left the specific agglutinins practically unaffected.

The converse experiment of saturating an Aertrycke "Mutton" serum with the unknown bacillus yielded the same result.

Serum.	End point of agglutination titre for: Bacillus "X"	End point of agglutination titre for: *B. aertrycke* ("Mutton")
B. aertrycke ("Mutton")	25,000	20,000
Ditto absorbed with—		
1. *B. aertrycke* ("Mutton")	< 500	< 500
2. Bacillus "X"	< 500	< 500

If the validity of deductions obtained by means of the absorption test be admitted, it would appear that the antigenic properties of the bacillus under examination and the "Mutton" type of *B. aertrycke* are identical.

Amongst the organisms studied by Schütze, in the investigation already referred to, a strain of *B. psittacosis*, which he states was "presumably of avian origin," was examined and was classified by him as of the "Mutton" type.

Through the courtesy of Dr. Besredka, opportunity was afforded me of comparing the organism under investigation with Nocard's original strain of *B. psittacosis*, a culture of which he kindly sent me. No difference in absorptive capacity was distinguishable between them, and in their antigenic properties they appeared to be identical.

Serum.	End point of agglutination titre for Bacillus "X."	End point of agglutination titre for *B. psittacosis.*
Bacillus "X"	25,000	25,000
Ditto absorbed with—		
1. Bacillus "X"	< 500	< 500
2. *B. psittacosis*	< 500	< 500

Whilst it would thus appear that no diversity, other than source of origin, exists between *B. psittacosis* and *B. aertrycke* of the "Mutton" type, it must be admitted that the question of permanency of these finer serological differences amongst members of this group of organisms has not been definitely settled. It may, however, be mentioned in its favour that no variation in this respect has been noted in strains of the organisms cultured for several years.

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A METHOD FOR THE ESTIMATION OF UREA BY SOY-BEAN.

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NUMEROUS methods for the estimation of urea by means of soy-bean urease have been devised; the procedure described below is comparatively simple and gives remarkably accurate results with known amounts of urea. Some features of it to which attention may be directed are:

(1) The use of an alcoholic extract of serum without evaporation and resolution in water.

(2) The direct titration of ammonia without removal by an air-current.

(3) The method of titration.

DESCRIPTION OF METHOD FOR SERUM.

(1) *Preliminary treatment of serum.*—5 c.c. of serum and 10 c.c. of methylated spirit are measured into a centrifuge tube; the estimation is, of course, carried out in duplicate wherever possible. The mixture is well stirred with a glass rod and centrifuged; 10 c.c. of the supernatant fluid are transferred to a 250 c.c. Erlenmeyer flask (if a very little of the precipitate is drawn up as well no appreciable error is caused) and 30 c.c. of water (from a cylinder) and 15 drops of 0·2 per cent. methyl red in alcohol are added. Sufficient 0·1 N. H_2SO_4 is added to produce a deep rose-red colour (about 1·5 c.c. will be required); the mixture is boiled for a few seconds to expel CO_2, cooled, and the flask stoppered if the subsequent neutralisation is not carried out at once.

(2) *Preparation of colour-standard.*—40 c.c. boiled water (*i.e.* the same volume as that in the Erlenmeyer neglecting the 0·1 N. H_2SO_4) and 15 drops methyl red are placed in a beaker and the indicator brought to the neutral colour (buff) by 0·01 N. H_2SO_4 or NaOH; to get the exact colour it is usually necessary to take fractions of a drop from the burette-nozzle on to the side of the beaker. One must select a particular shade of buff and adhere to it in the use of this method.

(3) *Method of colour comparison.*—A comparator of the very simple type used in C. J. Martin's method of titrating plasma is employed. A cardboard box, about 8 × 5 × 3 in., has the floor replaced by white paper and two holes are made in the longer side to admit two test-tubes; these must be of equal calibre, and are marked at the 10 c.c. level. The test-tubes are made to hang clear of the lower side of the box by means of rings of rubber tubing placed around them above the level of the holes in the upper side; 10 c.c. of the water neutralised to methyl red are poured into one of the two test-tubes, which is then corked. The acid fluid obtained as under (1) above is nearly neutralised by addition of 0·1 N. NaOH, the neutralisation being then carried on with 0·01 N. NaOH. When the neutral point is seen to be approaching, 10 c.c. of the fluid are poured into the other test-tube, and a comparison made with the standard. The fluid is subsequently poured back into the flask, but it is well to keep it in the test-tube until after the next addition of alkali to the flask, as a reserve of more acid fluid is then at hand in case the neutral point be overshot. Comparisons are made thus until an exact match is obtained, which with a little practice is brought about very quickly; the point of match is seldom in doubt over a range of more than one or two drops 0·01 N. This method of titration has been found very useful for other purposes, *e.g.* the titration of urine (see a forthcoming paper by Davies, Haldane and the writer in the *Journal of Physiology*). Colour-changes which seem very vague when observed in a flask in the ordinary way may be found to give a very sharp point when the box is used.

(4) *Decomposition of urea.*—2 grm. powdered soy-bean are placed in 100 c.c. normal saline; the mixture is allowed to stand with occasional shaking for ten minutes or so and then filtered. The extract can be kept for several days in the ice-chest. Two "blank" flasks are then prepared, each containing 40 c.c. boiled water and 15 drops methyl red; the water is then brought to the same reaction as the standard in the manner described above. 5 c.c. bean extract are then added to every flask, the extract being measured with as much accuracy as the serum and spirit.

The flasks are at once corked and incubated for one hour; a water-bath at 42° C. has been used as a rule in this work, but incubation in the air at 37° C. was found to be sufficient.

(5) *Titration of ammonia.*—15 c.c. 0·01 N. H_2SO_4 are added to each flask (a further quantity being added if the reaction is not then strongly acid). The liquid is then boiled for a few seconds, cooled, and the flask corked.

A fresh colour-standard is made, corresponding to the volume now present in the flasks, with 55 c.c. water and 15 drops methyl red. The excess of acid in the flasks is then titrated with 0·01 N. NaOH in the manner described under (3) above, the mean result of the blanks being, of course, subtracted. 1 c.c. 0·01 N. ammonia = 0·3 mgrm. urea.

TESTS OF THE METHOD.

Urea re-crystallised from alcohol was used. Kjeldahl estimations on it showed the following results:

0·1534 grm. gave 71·05 mgrm. N.; calculated, 71·58 mgrm.

0·0530 grm. gave 24·57 mgrm. N.; calculated, 24·73 mgrm.

A number of estimations were carried out on solutions of urea before the colour-comparator described above came to be employed; the results (Table I) were promising, but were irregular and sometimes higher than the calculated amounts. This was due, no doubt, to difficulty in determination of the exact end-point by the ordinary method of titration.

TABLE I.

Urea solution A,	C.c. 0·01 N. ammonia.	
0·1040 grm. in 200 c.c.:	Calculated.	Found.
10 c.c.	17·33	17·10, 17·25.
5 c.c.	8·66	8·70, 8·55, 8·50, 8·60, 8·40, 8·35, 8·70, 8·50.
3 c.c.	5·20	5·30.

When the comparator was used the results became more uniform (Table II); estimations were then made upon urea added to serum and cerebro-spinal fluid.

TABLE II.

Urea solution B,	C.c. 0·01 N. ammonia.		
0·0912 grm. in 200 c.c.:	Calculated.	Found.	
5 c.c.	7·60	7·50, 7·55, 7·55, 7·70.	
Urea solution C,			
0·1472 grm. in 250 c.c.:			
5 c.c.	9·80	9·55, 9·45, 9·45, 9·45.	
5 c.c.; incubation in 25 per cent. spirit	—	9·45, 9·45 = 96·4 per cent.	
3 c.c.	5·88	5·75, 5·75, 5·65.	
3 c.c. added to alcoholic extract of serum	—	5·75, 5·75 = 97·7 per cent.	
2 c.c. added to cerebro-spinal fluid	3·92	3·87, 3·92.	
5 c.c. added to 10 c.c. serum (estimation by method as described for serum):			
(1) With serum I.	3·27 (= ⅓ of 5 c.c.)	3·15, 3·25, 3·20, 3·10	mean 3·173 = 97 per cent.
(2) With serum II	—	3·12, 3·22	

It may be well to give the details of one of the last experiments given in the table above.

Two mixtures, A and B, were made as follows:

	A	B
Serum	10 c.c.	10 c.c.
Spirit	30 c.c.	30 c.c.
Urea solution	5 c.c.	Water 5 c.c.

Two portions of 15 c.c. were taken from each supernatant fluid, added to 35 c.c. water, and the estimation carried out in the manner given at the beginning of this paper. The results were as follows:

	C.c. 0·01 N. ammonia.		
Blanks	1·20	1·25 mean 1·23;	this has been subtracted from results A and B below.
B	3·47	3·52 mean 3·50	
A	6·72	6·62	
Subtract B	3·50	3·50	
Urea recovered	3·22	3·12 mean 3·17 = 0·951 mgrm. urea.	
Urea calculated	3·27=0·981 mgrm. urea.		

NOTES ON METHOD.

The degree of accuracy of these results caused some surprise, as the technique described is, at first sight, open to numerous objections. Thus:

(1) No buffer solution is used, as in van Slyke's method, to prevent injury of the enzyme by the ammonia. Evidently the very minute amount present is not sufficient to have any such action; in an estimation on a normal serum as described above the concentration of ammonia would be less than 0·001 N.

(2) It might seem that the loss of ammonia which must occur when the flask is opened would be appreciable. 1 grm. of water at 42° C. and 760 mm. absorbs 0·29 grm. ammonia (Roscoe and Dittmar [1]); this concentration is nearly 20 N. The conditions of such a determination, in which the water is exposed to an atmosphere of pure ammonia, are, of course, very different from those of the method here described; but the result is quoted to show the very high solubility of the gas in water even at 42° C.

(3) In the final colour comparison the fluid titrated is turbid (owing to coagulated protein from the bean-extract and lipoid from the serum), while the standard is clear; but with a little practice the turbidity does not cause any serious difficulty.

(4) The 0·01 N. NaOH has been made, with boiled water, from a 0·1 N. solution prepared without any precautions against the presence of carbonate. But the results given in the table seem to show that the troublesome process of preparing CO_2-free alkali can in this case be dispensed with.

(5) The high "blank" value, which may amount to one-third of the total result, may seem undesirable, but the figures given in the table show that this does not affect the accuracy of the results. Duplicate blanks should not differ by more than 0·1 c.c. 0·01 N. The "blank" value is usually from 1·0 to 1·30 c.c. for 5 c.c. extract; it varies with different extracts and with the length of time that the extract is kept.

Other indicators were tried (neutral red, phenol red) but methyl red was found to give better end-points. The use of the alcoholic extract of serum, without evaporation, is a great advantage. Firstly, time is saved; secondly, if such an extract is evaporated to, or nearly to, dryness, a white residue is obtained which is not readily soluble in water, or water and alcohol, even on heating. When one attempts to neutralise this suspension, the results are very variable. No satisfactory results were obtained in this work until evaporation of the alcohol was abandoned. The results given in Table II show that even 25 per cent. spirit has no adverse effect upon the enzyme; while the proportion present in the method as described is not more than 14 per cent.*

Cerebro-spinal fluids have been examined by this method without any preliminary treatment with alcohol, from 0·4 to 10 c.c. of the fluid being added to 30 c.c. water. A fluid containing a large amount of protein could, of course, be treated like a serum. When an estimation is made on urine, the amount of urea present can be foreseen roughly from the specific gravity; 1 or 2 c.c. of a 1 in 10 or 1 in 20 dilution is then taken. In the selection of the most suitable amounts of serum or cerebro-spinal fluid for estimation there is bound to be difficulty with a substance of which the amount present may be anything from 10 to 800 mgrm. per cent.; these fluids may contain more urea than does a dilute urine. The method seems to give the most accurate results when from 3 to 6 c.c. 0·01 N. ammonia is produced. Estimations on 5 c.c. of uræmic cerebro-spinal fluid have been made in which 90 c.c. was obtained, but the agreement between duplicates is then not so close; in such a case one must use 0·1 N. acid after incubation. 5 c.c. of serum has been recommended in the account of the method because this is simple both for measurement and calculation, but smaller amounts even of normal serum are sufficient; thus if 3 c.c. of a serum containing 30 mgrm. urea per cent. were mixed with 12 c.c. spirit, and 10 c.c. taken from this, 2 c.c. 0·01 N. ammonia would be obtained, which could be estimated with accuracy sufficient for most purposes.

Serum has been used in this investigation because it is so much more easily obtained, but the method can be applied equally well to whole blood. Serum will, of course, contain a higher percentage of urea than the whole blood. Suppose a blood to contain 26 mgrm. of urea in 100 grm. and to have the composition given by Schmidt (3), namely

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* Between 30 and 40 per cent. of the theoretical amount of ammonia is produced from urea by bean-extract even in the presence of 80 per cent. of alcohol (2).

$$1000\text{ grm. blood}\begin{cases}513\text{ grm. corpuscles with }350\text{ grm. water}\\ 487\text{ grm. plasma with }439\text{ grm. water}\end{cases}$$

the red corpuscles being completely permeable to urea, 100 grm. of the serum of this blood will contain 29·6 mgrm. of urea. In view of the wide range of urea-content even in normal persons, it would seem then to be for most purposes unimportant whether serum or blood is used.

The following are some examples of results obtained by the method here described (Table III).

Table III.

		Urea per 100 c.c.
Serum, healthy person		38·5 mgrm.
Plasma, case of cyanosis		24 ,,
Pooled Wassermann sera	A	34·5 ,,
,,	B	29·8 ,,
,,	C	27·5 ,,
Serum, nephritis of pregnancy		54 ,,
Ascitic fluid, peritoneal tumour		18 ,,
Œdema fluid, chronic nephritis		47·1 ,,
Cerebro-spinal fluid, uræmia	Case I: In life	153 ,,
,, ,,	Case II: *Post-mortem*	543 ,,
Case, uncertain diagnosis, low protein intake	Serum	18 ,,
	Cerebro-spinal fluid	11 ,,

THE UREA CONTENT OF NORMAL BLOOD.

Some recent data on this matter, none of them obtained by hypobromite methods, are brought together in Table IV; no restriction of diet was enforced except as stated in the table. It appears that amounts of from 15 mgrm. up to 60 mgrm. per 100 c.c. may occur in persons considered to be perfectly

Table IV.—*Urea Content of Normal Blood.*

	Subjects.	Urea, mgrm. per 100 c.c. blood.	Authors.
(1)	16 medical students and instructors, "strictly normal"	23·5 to 27·9 (per 100 grm.)	Folin and Denis (4).
(2)	17 patients "showing no disturbance of renal function," ages 14 to 58	16·5 to 50·3	Farr and Austin (5).
(3)	30 "normal men and women in robust health," ages 17 to 60, blood taken 3 hours after a standard meal	21·4 to 55·7	Gettler and Baker (6).
(4)	10 "perfectly normal adults," ages 20 to 40	21·4 to 49·3	McLean and Selling (7).

Subjects.	Urea, mgrm. per 100 c.c. blood.	Authors.
(5) "Young healthy adults," ages 20 to 35—		
(*a*) 28 persons on same diet	15·6 to 43·8	Addis and Watanabe (8)
(*b*) 106 estimations on 22 persons on unrestricted diet	22·5 to 60·0	
(*c*) 50 estimations on 1 person (A.) on unrestricted diet	24·5 to 60·0	
(*d*) 11 estimations on 1 person (M.) on unrestricted diet	27·0 to 51·0	
(6) 9 nurses and patients, "no subjects presenting evidence of metabolic disorder"	22·7 to 53·8 (one case, 64 to 85)	Hammett (9).

healthy. (One case in Hammett's series showed distinctly higher amounts: these are placed in brackets in the table.) It is noteworthy that the variations in a single person at different times are almost as great as those observed in a series of persons (Addis and Watanabe). Folin and Denis, in their early observations, seem to have happened upon a series in which the range was exceptionally narrow; the results of the five other investigations show fairly close agreement.

SUMMARY.

A technique for the estimation of urea by soy-bean is described by which amounts of 1 mgrm. can be estimated with an accuracy of 97 per cent. The ammonia produced is not removed by an air-current, and is titrated by means of a comparator. In the case of serum, an alcoholic extract is used without evaporation.

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THE VALUE OF THE VITAL-RED METHOD AS A CLINICAL MEANS FOR THE ESTIMATION OF THE VOLUME OF THE BLOOD.

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Many methods have been devised for the indirect determination of blood-volume, but few, unfortunately, can be applied to man. With the exception of those methods involving the inhalation of gases, they depend on the introduction of a known quantity of a foreign substance, *e.g.* a dye, into the blood-stream and the estimation of its dilution by the blood after a certain time. Time is allowed for mixing, but not long enough to permit appreciable escape of the dye from the circulation. From the standpoint of safety and simplicity vital red is probably the best substance which has been used so far for this purpose. This method is due to Keith, Rowntree and Geraghty, in whose paper (1) may also be found a good discussion of the older methods. The blood-volume was determined in this way by Keith (2) in cases of wound shock. The method yields values which are higher than those obtained by older methods, yet notwithstanding this, the results in wound shock (2) were found to be below the normal of the standard of even the old methods, thus emphasising the great drop in the volume of the circulating fluid in secondary shock.

The following investigation, suggested to me by Professor Starling, was carried out to test the reliability of the vital-red method for the clinical estimation of blood-volume.

The factors which are likely to disturb the absolute value of the result obtained by this means were studied under the following heads: (i) the uniformity of the dissemination of the dye throughout the whole of the circulating fluid, and the time taken for dissemination; (ii) the degree of the retention of dye by the plasma to the exclusion of the cells of the blood; (iii) the disappearance of the dye from the circulating fluid.

The animals used were cats and dogs; the latter possess the double advantage of a larger body-weight and a relative volume of plasma to corpuscles similar to that in man (57 per cent. : 43 per cent.).

Anæsthesia was induced by inhalation of a mixture of equal parts of chloroform and ether; to minimise the hæmolytic effect of the chloroform and ether, the maximum of analgesia was attained by a large dose of morphia given hypodermically about ten minutes before the chloroform and ether.

TECHNIQUE OF THE VITAL-RED METHOD.

(i) An initial sample (10 c.c.) of blood is drawn from a large vein into a paraffined centrifuge tube dusted inside with finely powdered potassium oxalate. This provides plasma for making up the standard for colorimetry and a sample of blood for the hæmatocrite.

(ii) A measured volume (0·2 c.c./1 kilo) of a 1·5 per cent. aqueous solution of vital red in a syringe is slowly (1 minute) introduced into a large vein. This may be effected by vene-puncture, or by inserting the needle into the rubber tubing connecting a burette to a cannula in the vein and then washing the dye in with gum-saline from the burette. Both methods were employed; the latter was found more convenient in cats.

(iii) Time is allowed for the dye to mingle with the circulating fluid (3 to 5 minutes, Keith) and a sample (10 c.c.) of dyed blood is drawn from a different vein into a pipette fitted with a venepuncture needle, which has previously been rinsed out with 1·6 per cent. sodium oxalate (3) to prevent clotting and hæmolysis; the blood is delivered into a prepared centrifuge tube. When repeated samples, *e.g.* every half minute, are required a cannula is inserted into the distal end of a vein or the proximal end of an artery, the first part of each sample being rejected.

The centrifuge tubes containing the blood are inverted half a dozen times so as to diffuse the oxalate for the prevention of clotting. They are then centrifuged at high speed (3000 revs./min.) for 20 minutes.

Some of the initial blood (i) having been drawn into the hæmatocrite tubes these are centrifuged at the highest possible speed for 2 to 3 minutes, until the corpuscles form a perfectly translucent mass.

(iv) A dilute solution of the dye is made up by diluting 1 c.c. (measured in a standard pipette) of the injected solution to 200 c.c. with saline in a graduated flask.

A small pipette of about 2 to 3 c.c. capacity is made, unit volume is etched on it, and it is fitted with a rubber teat. The following solutions are then made for comparison with the Dubosq colorimeter:

The standard.	
Plasma	1 vol.
Saline	1 ,,
Diluted dye solution ($\frac{1}{200}$)	1 ,,
Saline	1 ,,

Sample after injection of dye.	
Dyed plasma	1 vol.
Saline	3 vols.

Let r = ratio of strength of dye in sample to that of the standard,

then $r \times \frac{1}{200}$ = strength of dye solution in sample.

If v = vol. of dye solution injected,

and x = vol. of plasma,

$$\text{then } \frac{v}{x} = \frac{r}{200},$$

$$\therefore x = \frac{200 \times v}{r}.$$

Thus—

$$\text{volume of plasma} = \frac{\text{dilution factor} \times \text{no. of c.c. dye injected}}{\text{ratio of strength of dye in plasma}}$$

also—

$$\text{volume of blood} = \text{vol. of plasma} \times \frac{100 \text{ per cent.}}{\text{per cent. plasma by hæmatocrite}}$$

and—

$$\text{weight of blood} = \text{volume of blood} \times \text{sp. g.}$$

TECHNIQUE OF THE CONTROL METHOD.

The vital-red method was checked by methods involving either the reduction of the total blood by a known volume by bleeding or by increasing the circulating fluid by the intravenous infusion of gum-saline (Bayliss' fluid). The old method of bleeding and washing out the vessels was abandoned on account of the uncertainty of opening up all the stagnant capillary areas which one meets too often in histological technique; the experiments performed by this method showed the result to be only slightly below that obtained by the method about to be described:

Comparison.	Cat. 1.	Dogs: Average.
Bleeding and washing out blood-vessels . . .	168 c.c. .	(Two) 6·8 per cent. body-weight.
Hæmorrhage to the limit of existence . . .	175 c.c. .	(Six) 7·0 per cent. body-weight.

An initial sample of blood is taken for the colorimetric standard; 1 c.c. is carefully measured in a pipette and introduced into 199 c.c. of distilled water in a flask, coal-gas is bubbled through and the flask is tightly corked. The animal is now bled from a cannula placed in the femoral artery into a graduated cylinder containing oxalate, whilst warm gum-saline from a burette is introduced at the same rate into the external jugular vein. By this means quite nine-tenths of the hæmoglobin can be removed before the animal dies. When the animal shows signs of failure, the bleeding and gum-saline infusion are arrested. Time (3 to 5 minutes) is allowed for the thorough mixing of the remaining blood and gum-saline, and then a sample (referred to as the "ante-mortem sample") is taken for colour comparison, enabling one to estimate the hæmoglobin left in the vessels. By the infusion of gum-saline at the same rate and in the same amount as the blood withdrawn the volume of the circulating fluid is kept practically constant (4).

Records of the arterial blood-pressure in the carotid showed the blood-pressure to be maintained at a constant level under these conditions, notwithstanding the enormous dilution of the hæmoglobin.

The initial sample, the mixed drawn blood (oxalated) and the last ante-mortem sample are compared as carboxy-hæmoglobin in the Dubosq colorimeter. The blood-volume can then be calculated:

If s = c.c. in first sample and 100 per cent. = per cent. Hb. in s;
b = ,, by bleeding and β = per cent. Hb. in b;
and a = per cent. Hb. in the last ante-mortem sample; then—
$100-a$ = per cent. Hb. lost by the animal during bleeding.

$$\therefore \text{ total volume of blood} = \left(s + b\,\frac{\beta}{100}\right) \times \frac{100}{100 - a}.$$

The following example illustrates this:

Dog 2.—i.	Initial sample	1 c.c.	100 per cent. Hb.
ii.	Hæmorrhage	1350 ,,	37 ,, Hb.
iii.	A-M sample	1 ,,	9 ,, Hb.

$$\begin{aligned} \therefore \text{(i)} + \text{(ii)} = 91 \text{ per cent. of the blood} &\equiv 1 \text{ c.c. @ } 100 \text{ per cent. Hb.} \\ &\quad + 1350 \text{ c.c. @ } 37 \text{ per cent. Hb.} \\ &\equiv (1 + 499) \text{ c.c. @ } 100 \text{ per cent. Hb.} \\ &\equiv 500 \text{ c.c.} \end{aligned}$$

$$\therefore 100 \text{ per cent. of the blood} = 500 \times \frac{100}{91} = 550 \text{ c.c.}$$

The dilution of corpuscles was also used as a check on the Hb. method in those cases where there was no trace of hæmolysis; the agreement was found to be a very close one:

Blood-volume (weight) expressed as a percentage of body-weight.

By Hb. dilution . . . = 7·04 per cent. (average for 6 dogs).
,, corpuscular dilution = 7·00 ,, ,, ,, 9 ,,

This method of maximal dilution of the blood *in vivo* yields, in the writer's opinion, the nearest approach hitherto attained to the absolute value of the blood-volume, and is therefore used as a basis of comparison for the other methods, a factor being determined in each of the other methods which is necessary to convert the observed result into the true one. Even measurements of the concentration of dye in the circulation may yield, under certain experimental conditions in animals, values much nearer the true than the usual vital-red technique. Thus, simultaneous bleeding and accurate replace-ment of fluid by infusion of gum-saline in about one hour's time after the injection of large doses of dye gave the following results :

	Dye.	Blood withdrawn. =gum saline infused.	Blood-weight.	
			Calculated from dilution of dye.	Bleeding method.
Dog 9	Vital-red 30 mgrm./kilo.	160 c.c. 400 c.c.	6·77 per cent. 6·65 per cent.	6·43 per cent.
Dog 10	Congo-red 20 mgrm./kilo.	300 c.c.	7·3 per cent.	7·2 per cent.

DISTURBING FACTORS IN THE VITAL-RED TECHNIQUE.

(i) *The optimum interval for withdrawal of blood* after injection of the dye was found to be limited on the one hand by the blood-flow and on the other hand by the disappearance of the dye from the circulation. When the anæsthesia was deep the blood-volume yielded a low figure, indicating a small circulation for the dye, probably because some capillary areas were closed to the dye. On applying massage and passive movements the 4-minute interval yielded a much higher value, as shown by the following two cats of nearly equal weight—3·8 and 3·75 kilos.:

	Blood-volume as percentage of body-weight.	
	Vital red.	Bleeding.
Cat 1	6·0 per cent.	4·77
Cat 2—massage and movement	6·6 ,,	4·85
Difference	0·60	0·08

The rate of the dissemination of the dye in the blood-stream was determined by injecting the dye into the proximal end of the left external jugular vein and withdrawing samples of blood every half minute from the distal end of the right external jugular vein. Curve 1 shows that the dye reaches its maximum concentration in the general circulation in the third half minute; interpolation gives it as eighty seconds—a period which is about twice the total circulation time (5) for a dog of this size (9¼ kilos.). Probably twice the total circulation time is long enough for thorough admixture of the dye in man, in which case 2–2½ minutes would be the optimum time for the withdrawal of blood. The way in which the time of withdrawal after injection of the vital red affects the result is shown in—

Dog 13: 9¼ kilos., 8 mgrm. dye/kilo., 1 *or* 1½ *minute sample.*
Blood-volume (weight) as a percentage of body-weight :
Vital-red method = 8·8 per cent.
Bleeding . . = 7·9 ,,

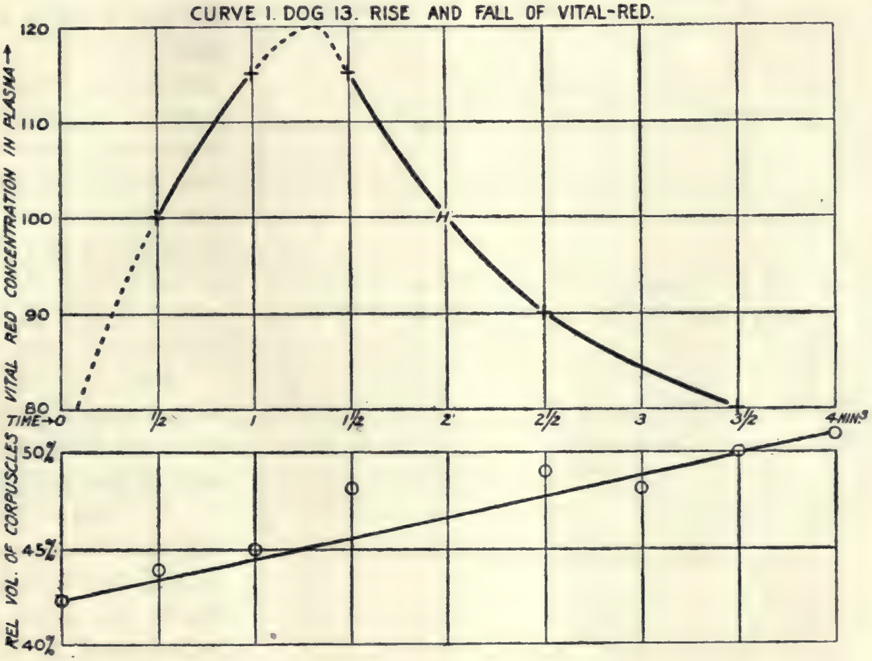
$$\left.\begin{array}{l}\text{which give the factor for correcting}\\ \quad\text{the vital-red result}\end{array}\right\} = \frac{7{\cdot}9}{8{\cdot}8} = {\cdot}90.$$

Whereas—

the average conversion factor for six other dogs on a 4-*minute* sample = ·78.

This experiment shows an interesting rise in the relative corpuscular volume with hæmorrhage. Another experiment was performed to determine whether this was an actual concentration of corpuscles or was merely an imbibition of fluid by the corpuscles from the plasma.

CURVE 1. DOG 13. RISE AND FALL OF VITAL-RED.

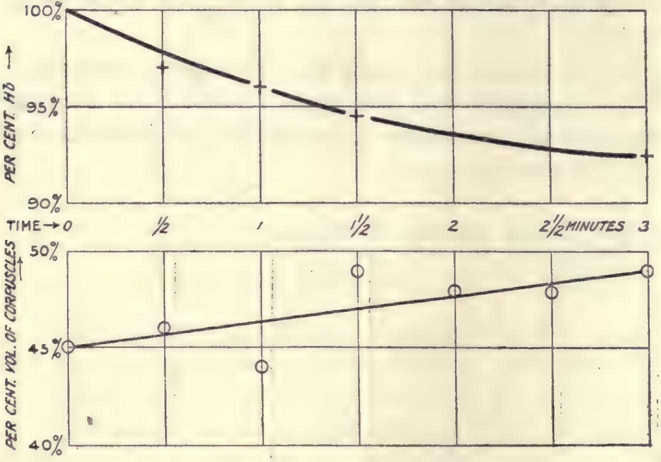


The gradual fall in percentage of hæmoglobin in spite of the rise in the percentage volume of corpuscles proves the swelling of the corpuscles as seen in Curve 2.

This may be a local phenomenon occurring only at the site of the hæmorrhage where there is a sudden drop in the pressure to which the corpuscle is exposed, or it may be a general condition, representing the first stage in the rapid withdrawal of fluid from the tissues. It may also be a defensive mechanism to minimise the loss of blood from the wound by increasing the viscosity of the blood.

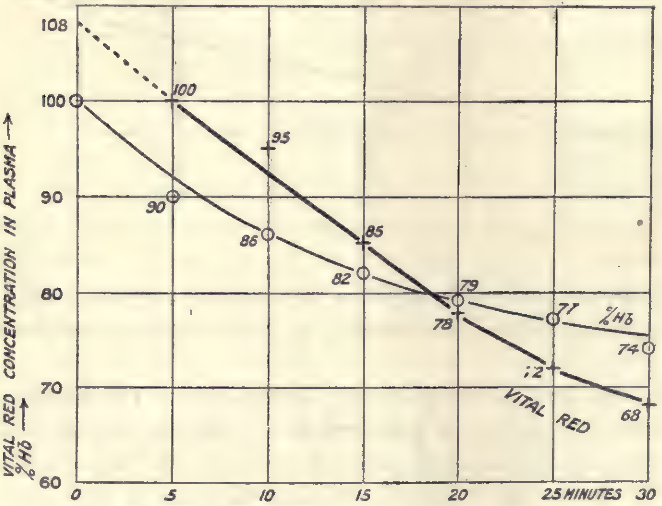
Hooper, Smith, Belt and Whipple (3), using brilliant vital red, recently found that the concentration of this dye at the end of 2 minutes and 4 minutes was practically the same (100 : 99·9), and they state that the "blood-volume estimations based on samples collected at the 4-minute interval cannot be invalidated because of the elimination of dye from circulation." The equality of these concentrations may equally well be accounted for by leakage from the beginning, their first point (100) being on the up curve and their second point (99·9) on the down curve (compare Curve 1); it is probable that the elimination of any non-toxic dye varies with its concentration in the blood-stream, and is therefore at a maximum at the very beginning. Their high result for the amount of blood in dogs (10 per cent. body-weight) may be due to the loss of dye in the first 4 minutes.

CURVE 2 DOG 15 SWELLING OF CORPUSCLES WITH HÆMORRHAGE



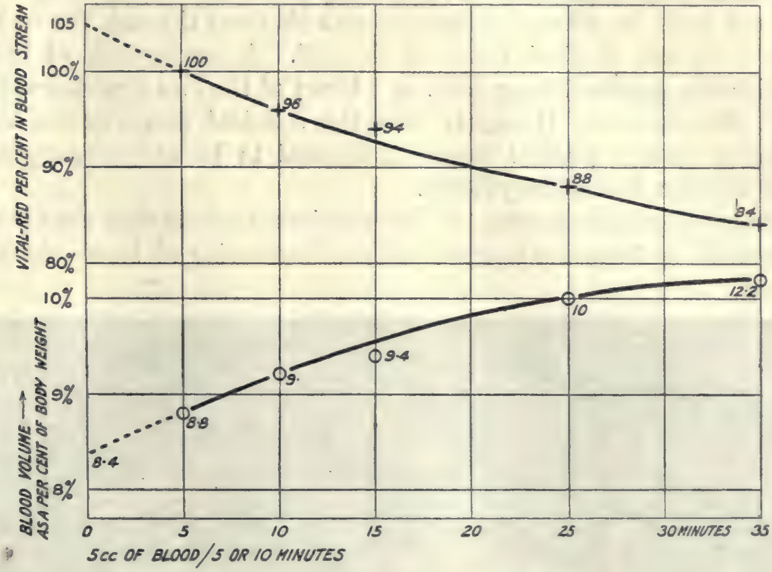
The rate of disappearance of vital red from the circulation is seen in Curves 3, 4 and 5. The loss of fluid in the case of this smaller animal in Curve 3 was made up, after each withdrawal of blood, by the intravenous infusion of an equal volume of gum-saline. The repeated small hæmorrhages lower the concentration of the dye at a faster rate than the fall in percentage of Hb., proving conclusively that the dye leaves the circulating plasma. The way in which this leakage vitiates the results of the calculated blood-volume is shown in Curve 4, in which the dog makes up the loss of fluid by drawing on his tissues. The result is seen to rise

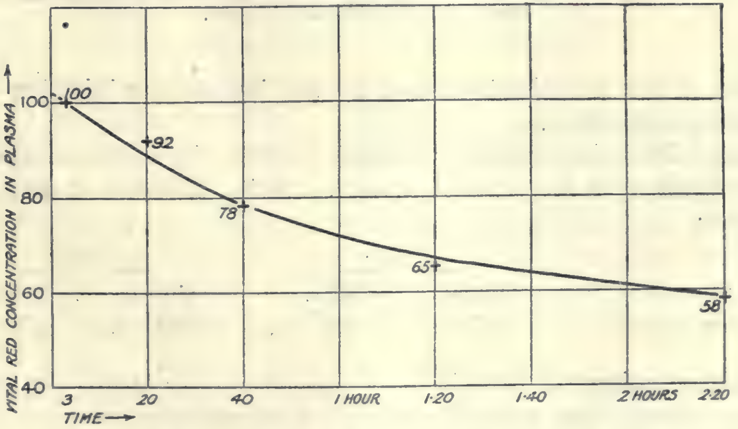
CURVE 3 CAT I DISAPPEARANCE OF VITAL-RED FROM THE BLOOD STREAM



7cc OF BLOOD / 5 MINUTES
AND REPLACED BY 5cc GUM SALINE

steadily as time passes, and the value obtained by extrapolation (8·35 per cent.) is no doubt nearer the true blood-volume than the value (8·8 per cent. of the body-weight) given by the usual vital-red technique. The percentage of hæmoglobin was estimated both as carboxy-hæmoglobin and as acid-hæmatin; the purity and depth of colour in the latter method was found to be more than counterbalanced by the tendency of the acid-hæmatin particles to aggregation and precipitation. The carboxy-hæmoglobin still remains, as Boycott has shown, the best method for the colorimetric estimation of hæmoglobin,

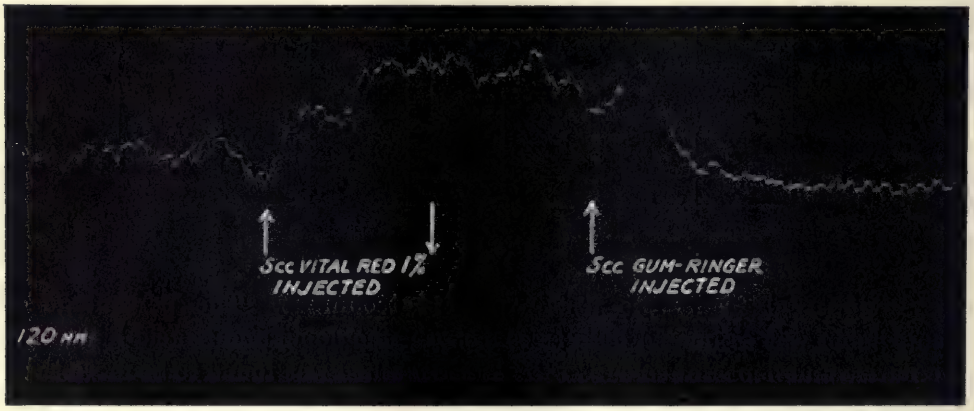

CURVE 4 FALL IN VITAL-RED CONCENTRATION AND RISE IN BLOOD VOLUME RESULT


CURVE 5. DOG 4. DISAPPEARANCE OF VITAL-RED FROM THE BLOOD STREAM

especially in the comparison of a large number of samples of very different dilutions.

In Curve 5 the fall was observed at longer intervals, the concentration of dye in the circulating plasma falling to 72 per cent. in 1 hour. The sample of vital red used in these experiments was obtained through the courtesy of Dr. Dale from the Medical Research Council. A comparison of the above curves with the results (77 per cent. in 1 hour) of the two experiments quoted by Keith, Rowntree and Geraghty show the colloidal properties of the vital red prepared by the Medical Research Council to be only slightly different from the German dye used by Keith.

Tracings taken during some of the experiments show that vital red causes only the slight and transient physiological effects—rise of blood-pressure and quickening of the heart-beat—caused by the intravenous injection of almost any innocuous substance.



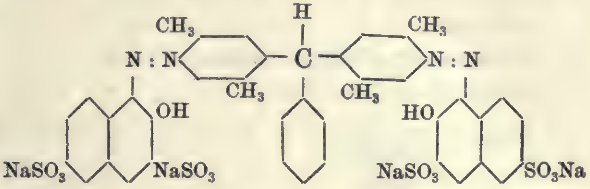
Tracing 1.—Cat 1 (3·8 kilos.). Tracing showing temporary rise of blood-pressure caused by intravenous injection of vital red.

Repeated determinations are rendered valueless by the curvilinear fall in the concentration of the circulating dye. This is shown in the following estimations:

	Dog 1.		Dog 4.	
Time	4 min.	1 hr.	4 min.	3 hr. 40 min.
Plasma volume	237 c.c.	296 c.c.	200 c.c.	277 c.c.

Re-determination of the blood-volume, therefore, cannot be performed while any appreciable amount of the dye remains in the circulation. Smith (6) recently described a method for repeated determinations in the same animal at short intervals in which the error does not exceed 5 per cent.

(ii) *The degree of the retention of the dye by the plasma to the exclusion of* *the cells of the blood.*—Vital red is disodium disulphonaphthol-azo-tetramethyl-



triphenyl-methane, hence the colloidal particles of the acid dye should carry negative charges. This was verified by kataphoresis in Hardy's (7) modification of Whetham's apparatus when vital red was found to dissociate so that the coloured particles moved to the anode. Now the charge on hæmoglobin is electro-positive, suggesting an affinity for the dye. A preliminary experiment was performed to determine whether the intact corpuscles also carried a positive charge. On examination in an electric field by a method described elsewhere (8) the corpuscles in freshly drawn blood were found to carry the same charge as the contained hæmoglobin.*

Experiments were then performed to determine whether the electro-negative dye was adsorbed to any appreciable extent by the electro-positive corpuscles.

Experiment 1—on fresh oxalated dog's blood:

(i) Determine the relative volume of corpuscles and plasma with the hæmatocrite.

Let p = percentage of plasma.

(ii) Place $\left\{\begin{array}{l}\text{24 c.c. whole blood}\\ \text{1 c.c. vital red solution}\end{array}\right\}$ in a centrifuge tube.

Invert gently and repeatedly for 5 minutes.
Centrifuge for 20 minutes at 3000 revs./min.
Pipette off the stained plasma.

$$\left.\begin{array}{r}\text{Then dilution of dye}\\ \text{if in plasma } \textit{only}\end{array}\right\} = \frac{100}{1 + \cdot 24\,p} \text{ per cent. } (\textit{by calculation}).$$

(iii) Centrifuge 25 c.c. of whole blood and pipette off the plasma. To 9 c.c. plasma add 1 c.c. of the vital red solution. Then dilution of dye = 10 per cent., and this is used as a standard of comparison for the actual strength of dye in the plasma from (ii), by means of the Dubosq colorimeter.

---

* Since the above was written Prof. Bayliss has found that corpuscles suspended in isotonic sugar solution, when examined in bulk in Whetham's apparatus, carry negative charges, *i.e.* opposite to what was found microscopically in whole blood in contact with glass (7). This brings the corpuscular envelopes into line with other tissue cells and renders intelligible the impermeability to the electro-negative dye vital red as well as the Hamburger effect.

Results :

Taking the strength found by calculation for (ii) as	100 per cent.
The colorimeter gave the actual dye in plasma of (ii) as	98·4 ,,
Hence : dye taken up by corpuscles =	1·6 ,,

(A value within the limits of error in colorimetric comparisons.)

Experiment 2.—To 10 c.c. of blood add 5 c.c. dye solution.

Centrifuge at high speed for 40 minutes.

The layer of white corpuscles resting on the erythrocytes appeared deeply stained, yet on microscopical examination neither the nucleus nor protoplasm was found to contain the dye.

As similar results were found by Keith (1) it was not considered necessary to carry out any further experiments in this direction.

(iii) *Elimination of the dye.*—For a study of the leakage of vital red from the circulating plasma, lymph and urine were collected during and after the injection of the dye. To facilitate the introduction of cannulæ into the thoracic duct and into the ureters of small as well as large animals a cannula introducer (Fig. 1) was devised.

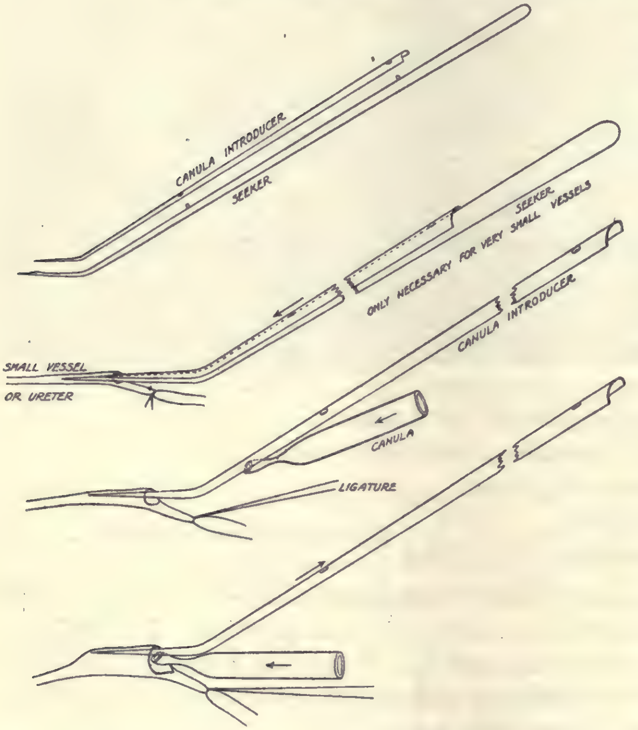


Fig. 1.—Cannula Introducer. (To be obtained from A. E. Braid & Co., Ltd., 30, Gower Place, London, W.C. 1.)

Dogs only were used in this group of experiments ; they were placed under chloroform and ether anæsthesia after a preliminary dose of morphia. A cannula was placed in the thoracic duct and in each ureter, the ureters being exposed extra-peritoneally.

About ten times the usual clinical dose (3 mgrm./kilo.) of vital red was injected into the external jugular vein. The dye appeared in the ureter first (five minutes), later in the thoracic duct (ten minutes), the difference being accounted for by the longer passage presented to the dye in the latter case after leaving the blood-vessels. In order to increase the lymph and urinary flow warm gum-saline was introduced into the same vein.

At the end of an hour a comparison of the strengths of dye was made as shown in the following results:

		Colorimeter.	Concentration.
Plasma	At 5 minutes	100 per cent.	
	At 1 hour	50 ,,	
	Average	(75)	1
Lymph		89 per cent.	1·2
Urine		356 ,,	4·7

(The high concentration of colouring matter in the urine may be explained by the presence of urinary pigments or by the resorption of fluid by the tubules.)

The rapid fall in the concentration of the dye in the circulating plasma—to one-half in one hour—is accounted for by the unusual supply of saline in this case by intravenous infusion for the promotion of an active flow of lymph. Under ordinary conditions the dye is not eliminated at half this rate (Table on p. 157) and is not so obvious in the bladder urine, the saline acting as the diuretic in the above experiment. The urine was found to obtain albumen in this experiment. Probably the best time for making a determination of the blood volume would be immediately before lunch, thus minimising the error due to elimination of the dye with the digestive secretions and urinary excretion.

COMPARISON OF METHODS.

Reference to the experimental results (on p. 154) shows that for the vital-red method it is necessary to multiply the result obtained by the dye method when taking a 4-minute sample—

in the case of	cats	by 0·78 (average of 2 cats).
	dogs	by 0·78 (average of 4 dogs).

As the factor appears to be identical for the two different species it is suggested that the same factor might be available for the correction of the very interesting figures published by Keith (1) of the blood-volume of man in various pathological conditions. Before this can be accurately determined it will be necessary to make due allowance for the slower circulation time in man on the basis of a 4-minute sample of vital-red plasma. As a first approximation, 0·8 may be taken as the correction factor, thus:

Average blood weight in man by the vital-red method (Keith, etc.) = 8·8 per cent. body-weight;

$$\text{Corrected value} \bumpeq 8{\cdot}8 \times 0{\cdot}8$$

$$\bumpeq 7 \text{ per cent. body-weight.}$$

It is interesting to note that in the two criminals who were bled to death

Bischoff found 7·6 per cent. as the average (the accepted standard 1858–1881) which is a little higher than the above value, probably because he washed out some myo-hæmatin from the muscles. Further, the corrected value (7 per cent.) occupies a mean position between the value (5 per cent.) obtained by the CO-method in the hands of Haldane and Lorrain Smith (9) and the vital-red method (8·8 per cent.) by Keith, etc. The low figure yielded by the CO inhalation and carmine titration method may be accounted for by the direct toxic action of CO on the vessel walls thus shutting off large capillary areas where circulation is at a standstill; moreover physiological substances other than hæmoglobin may show an affinity for CO (Barcroft).

Table showing the Results of Different Methods of estimating Blood-volume.

			Blood volume in c.c.			Blood = per cent. body-weight.		
No.	Weight in kilos.	Vital red mgrm./kilo.	Bleeding to death.	Hb. method.	Vital-red method.	True value.	Vital red.	Correction factor = true/dye.
				Cats.				
1	3·8	12	168	175	217	4·8	6·0	0·80
2	3·75	3	174	—	235	4·86	6·6 m	0·76
3	3·5	3	—	158	h	4·66	h	—
7	2·6	6	—	1·11	h	4·48	h	—
	(m = massage and movement.)							
					Average (cats)	4·7 %	6·3 %	0·78
				Dogs.				
1	6	3	395	—	530	6·98	9·39	0·75
2	8·75	3	550	—	690	6·65	8·37	0·79
3	8·5	17½	—	542	702	6·79	8·78	0·77
4	5	3	—	357	h	7·57	h	—
7	6	2½	—	408	h	7·13	h	—
8	9¾	2	—	682	844	7·38	9·20	0·80
	By the vital-red method (4-minute sample)—average (4 dogs)						8·9%	0·78
*9	5	30	—	304	628	6·43	*13·3	∵ very large dose
18	9¼	8	—	690	765	7·9	8·8	0·90
							1-minute sample	
					By bleeding—average (8 dogs)	7·1%		

* 6·7 per cent. from dilution of dye by hæmorrhage. (h = hæmolysis)

FURTHER STUDIES OF THE INDIRECT METHOD.

Hæmolysis, in however slight a degree, renders the vital-red test valueless —a complication more often met with in cats than in dogs. The ideal dye would be one, therefore, which would circulate in a form which has a reddish tint, thus giving rise to no peculiar colouring of the lips of the patient, and yet, after withdrawal from the circulation, could be converted chemically into a deep colour not to be confused with hæmoglobin. Congo red, which in the form of the sodium salt is red and in the form of the free acid is blue, was thought to fulfil these requirements. On putting this to the test of experiment it was found that on acidification of the dyed plasma the small dose of dye permissible in clinical cases was far too feeble for comparison even in the Dubosq colorimeter when used in this way. For checking the results in animals, dyes of various colours were tried, the methods of study being indicated below.

Electro-negative dyes.—As an example Congo-blue may be quoted. A 1·5 per cent. solution in distilled water was made and sterilised for 15 minutes at boiling-point. It was filtered through glass-wool whilst hot and twice again when cold. The solution showed no sedimentation after 24 hours.

A mixture of 1 c.c. 1·5 per cent. dye + 24 c.c. blood was put in a centrifuge tube and gently inverted periodically for 5 minutes. On centrifuging for 20 minutes at 3000 revs./min., although the corpuscles took up none of the dye (as one would expect from the positive charge on the corpuscles) yet hardly any of the dye remained suspended in the plasma. Thus Congo-blue, though possessing desirable indiffusibility, yet in solution occurs as colloidal aggregates too large for our purpose. Dawson, Evans and Whipple (10) have recently tested this dye in dogs and found very low readings in the plasma.

Electro-positive dyes.—Night-blue may be taken as an example. The solution was prepared as above. 1 c.c. of $\frac{3}{4}$ per cent. solution was injected into a dog; a sample of blood withdrawn 5 minutes later showed only a barely appreciable quantity of the dye. This electro-positive dye was probably adsorbed by the electro-negative tissues.

Other solvents.—The very complete indiffusibility of aniline blue was a clear recommendation for this dye, but the difficulty of obtaining the variety which is soluble in water prevented its use in the same way as vital red. When dissolved in ether, and though introduced into the circulation very slowly, yet its toxicity soon revealed itself. Weaker solutions were then tried, but the samples of plasma yielded were not found deep enough in colour for accurate comparison.

Histological dyes.—Of these, pyronin was found to be one of the best, but even this was inferior to vital red. They are mostly too diffusible.

Dawson, Evans and Whipple (10) have recently shown that a very large number of dyes possess the properties essential for determination of blood-volume. They find that a blue azo dye, T 1824 (ortho-toluidin with 2 molecules 1–8-amido 2–4-disulphonic acid), is slightly superior to vital red, especially as regards ease and accuracy of colorimetric readings.

Emulsions.—Various dyes were worked into a fine emulsion with petroleum but were found totally impracticable on account of viscosity and embolism, though the perfect indiffusibility of petroleum has much to commend it.

Suspensions.—Indian ink was evaporated over a water-bath to drive off the toxic terpenes. It was then worked into a fine paste with water and extracted several times. It was sterilised, filtered thrice through glass wool, and finally centrifuged to precipitate the coarser particles. 10 c.c. of the supernatant fluid were injected into a dog but hardly a trace was found 5 minutes later in the circulating blood; it may have undergone phagocytosis in the liver, etc.

Of all the dyes tested Congo-red seemed to possess most of the desired properties, and a fuller investigation of this dye was undertaken. It is a non-toxic, comparatively indiffusible dye to living tissues, and it has been shown by Prof. Bayliss to consist mainly of single molecules (11), and so does not sediment in the centrifuge tube. It possesses the great advantage over vital red that when the dyed plasma is being compared with the standard in the Dubosq colorimeter, the addition of a drop of ammonia to each clears up the fluid and brightens the Congo-red, thus making the comparison easier to the eye. The technique in all other respects is similar to that of the vital-red method. The following are the results obtained:

CATS.

No.	Weight in kilos.	Congo-red mgrm./kilo.	Blood-volume in c.c.		Blood = per cent. body-weight.		Conversion factor = true/dye.
			Bleeding.	Congo-red.	Bleeding.	Congo-red.	
4	3·0	17	133	156	4·66	5·49	0·85
5	1·75	8	—	92	—	5·50	—
6	3·0	5	—	156	—	5·49	—
					Average (3 cats)	5·5 per cent.	

DOGS.

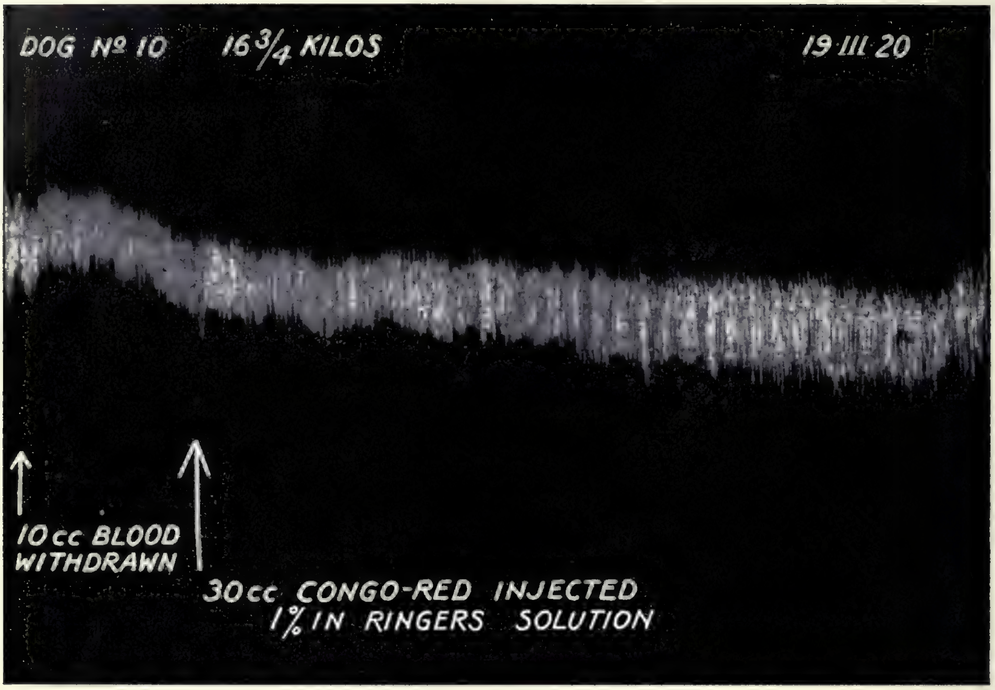
No.	Weight in kilos.	Congo-red mgrm./kilo.	Blood-volume in c.c.		Blood = per cent. body-weight.		Conversion factor = true/dye.
			Bleeding.	Congo-red.	Bleeding.	Congo-red.	
5	10·0	1½	—	662	—	7·02	—
6	7·7	·5	485	540	6·69	7·42	0·90
11	4·75	10	303	333	6·76	7·42	0·91
12	9·75	10	670	750	7·30	8·15	0·89
14	8·9	5	525	615	6·25	7·32	0·85
15	16·5	3	1200	1255	7·7	8·06	0·96
			By the Congo-red method—average			7·6 %	× 0·9
*10	16·75	20	1136	1750	7·2	*11·7	∵ very large dose.
			By bleeding—average		7·0 %		

* 7·3 per cent. from dilution of dye by hæmorrhage.

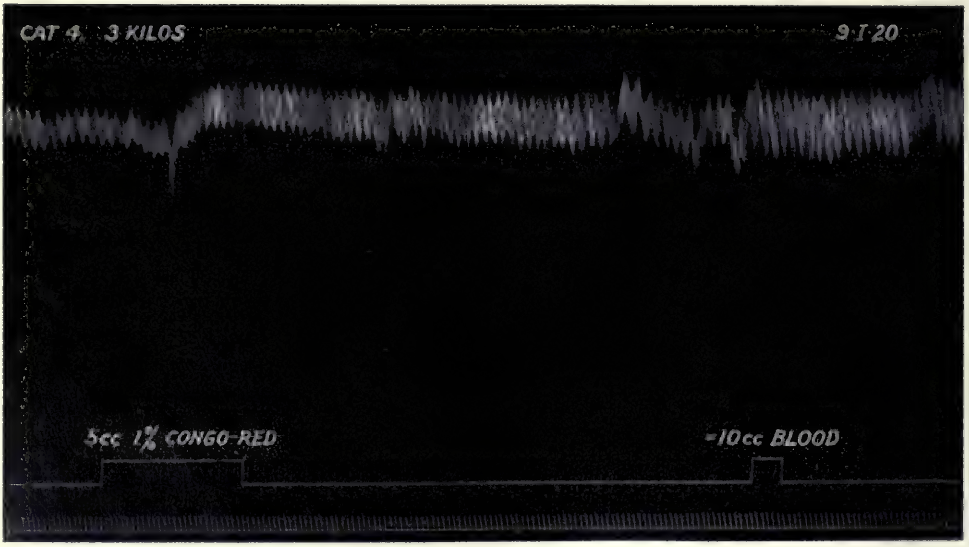
A comparison with the results obtained with vital red shows Congo-red to be very much nearer the true value as in the following analysis:

	Cats.		Dogs.	
	Blood-weight.	Correction factor.	Blood-weight.	Correction factor.
Bleeding	4·8 per cent.	× 1·0	7·0 per cent.	× 1·0
Congo-red	5·5 ,,	× 0·85	7·6 ,,	× 0·9
Vital red	6·3 ,,	× 0·78	8·9 ,,	× 0·78

The reason for this is shown in Curve 6, where the rate of disappearance



Tracing 2.



Tracing 3.

Harris. The Estimation of the Volume of the Blood.

of Congo-red (in a cat of 3 kilos.) is seen to be much less rapid than that of vital red (in a cat of 3·8 kilos.).

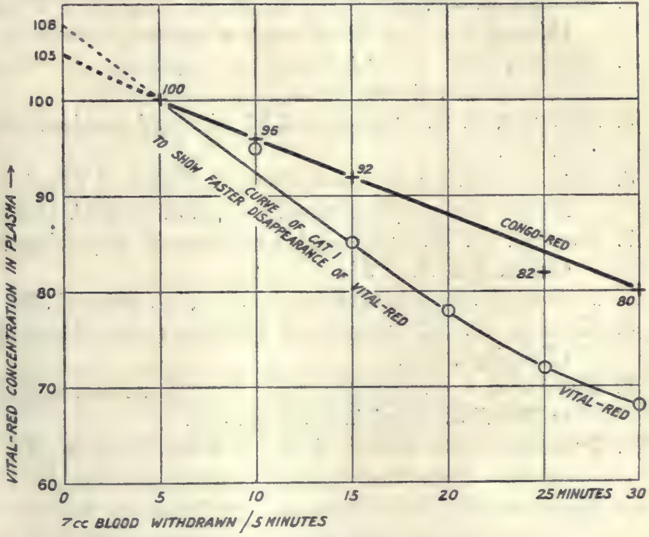
It appears, however, in the ureters and thoracic duct at about the same time after injection as does vital red:

	Thoracic duct.	Ureters.	
Vital red . .	7 min. .	11 min. .	(Dog 9).
Congo-red . .	8 min. .	10 min. .	(Dog 10).

The physiological action of Congo-red injected in the small doses required for these determinations is not appreciable; even in much larger doses the effect on the blood-pressure, pulse and respirations is only slight, as is shown in the tracings Nos. 2 and 3, in which five times the suggested clinical dose was injected.

So far, then, it appears that Congo-red may prove a better dye for the estimation of the blood-volume in man. Further investigation is being carried out.

CURVE 6. CAT 4. RATE OF DISAPPEARANCE OF CONGO-RED FROM THE BLOOD STREAM



CONCLUSIONS.

1. A control method is described for the determination of the blood-volume in animals which depends on removing the maximum amount of Hb. compatible with life whilst keeping the blood-volume constant by the simultaneous injection of gum-saline at the same rate.

2. The vital-red method gives too high a value because the dye leaves the blood-vessels even before it has spread evenly through the circulating fluid. The optimum interval for withdrawal of the blood sample is probably 2–2½ minutes.

3. The correction factor, to convert the vital-red result into the true value, was found to be 0·78 for cats and dogs for a 4-minute sample of dyed plasma. It was as high as 0·9 when a 1-minute sample was taken. It is suggested that the correction factor for man lies between × 0·8 and × 0·9 according to the time of withdrawal of the sample.

4. Congo-red was found to give results nearer the true value, the conversion factor for a 4-minute sample being × 0·9 for dogs.

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CRITICAL REVIEW.

THE ÆTIOLOGY OF INFLUENZA.

(*Concluded from No.* 2, *p*. 126.)

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THE RELATION OF FILTERABLE VIRUSES TO INFLUENZA.

WITH remarkable unanimity investigators in nearly every country have described an ultramicroscopic virus as the cause of influenza. This unanimity alone carries with it considerable weight, but it must be confessed that the trivial nature of most of the papers and the obvious lack of experience in some of the investigators, compels caution in accepting the claims. This caution will be understood to be all the more desirable when it is realised that the introduction of the invisible virus concept absolves the discoverers from the necessity of producing evidence of a morphologically characteristic microbe.

In Germany W. Kruse (1), in 1914, claimed as the result of one experiment that common colds are due to a filter-passing organism to which he gave the name *Aphanozoa coryzæ.* His assistant suffered from a cold. The nasal secretion diluted with saline was filtered through a Berkefeld filter and several drops of the filtrate were placed into the noses of thirty members of his class. Fifteen of these developed colds, while only one of the thirty-six uninoculated members of the class succumbed. It is obvious that since the assistant and one of the controls had colds this disease must have been epidemic at the time. The action of the filtrate may have been *nil* or it may have acted as a local irritant to the nasal mucous membrane like distilled water and so accounted for the colds. The author produced no evidence of the existence of a living virus in the nasal secretion at all. Exactly the same result might have been produced by placing formalin in the nose of persons exposed to infection. Therefore his attribution of colds to his so-called "*Aphanozoa coryzæ*" is totally unjustified.

In America Foster (23) repeated and extended the work of Kruse. He made filtrates of nasal secretions from acute colds in the same way as Kruse and dropped these into the anterior nares of ten soldiers. Seven out of these developed "clear-cut and definite symptoms of acute coryza." The clinical histories are given in detail and are too long to quote. There is no doubt that the men suffered from some nasal affection, but in our opinion none could be really described as having ordinary colds. If they had it may still be objected that they were not isolated in any way and were thus exposed to infection; in fact a relative of one developed a cold at the same time. The author next succeeded in growing the virus in Noguchi's medium,* the inocula being obtained from the filtered nasal secretions of eleven cases of natural colds and five cases of experimental colds. Subcultures of these cultures were obtained with ease. In twenty-four hours a positive growth was recognised by the development of an opalescent ring-like cloudiness above the hæmolytic area and around the fragment of kidney. The cloudiness after prolonged staining by Giemsa showed, under the microscope, minute coccoid bodies (illustrated). These bodies are accepted as microbes, the cloudiness in the tubes is evidence of growth, and the cultures applied to eleven further soldiers gave all "unmistakable symptoms of acute coryza."

In 1917 Dold (4), working in a German educational institute in Shanghai, carried out a similar experiment with similar results upon Chinese students, all of whom lived and slept together. He then inoculated forty Chinese hospital patients suffering from minor surgical diseases and all retained in bed. Fifteen other patients in the same ward were used as controls. In this experiment no one became ill. The difference in the results of the two experiments suggests that the students were living under conditions which rendered them liable to colds while the patients were not, and that the inoculation had no special bearing upon the incidence of the disease.

The author then proceeded to grow his invisible virus in Noguchi's medium. In two days growths identical to Foster's were obtained; these were propagated to the fourth generation. In stained preparations nothing could be seen, but under dark-ground illumination numerous minute particles in active movement were observed. This culture placed into the nostrils of three Chinese produced in two increased nasal secretion and slight cough.

The author thus claimed that he had discovered a characteristic virus as motile particles, that these particles were living because they were propagated in culture and that they reproduced their characteristic lesion in man.

On the other hand it will be manifest to anyone who has had experience of Noguchi's medium that the deductions of these authors must arouse suspicion. These growing opacities are to be observed often enough in uninoculated Noguchi tubes, and, of course, particles demonstrable by prolonged Giemsa staining may be found in most albuminous fluids. Further, the symptoms produced in man were not really very like the natural disease.

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* Noguchi's medium consists of unheated ascitic-fluid-broth covered with a layer of paraffin. At the bottom of the tube is placed a fragment of fresh rabbit's kidney. Ascitic fluid is extremely difficult to obtain sterile, in fact it is probably contaminated while still in the peritoneal cavity. These contaminations often do not develop for a number of days, and therefore very special care is necessary to be certain that this medium is sterile. Noguchi's medium is selected by workers upon filter-passers, because, by means of it, Noguchi grew the "globoid bodies" which he advanced as the parasites of poliomyelitis and rabies. In this respect, however, his work is not universally accepted.

We are of opinion that the fact of the presence of these natural opacities makes the whole argument of these authors precarious, and the other evidence adduced is not sufficiently strong to have much weight against this objection. Thus, for the present, we have no reason to believe that common colds are due to filter-passing microbes.

We have reviewed the history of the attempt to associate invisible viruses with colds, because the claims and experimental results adduced are very similar to or in some cases identical to those associating invisible viruses with influenza. Kruse suggested that influenza was due to an invisible virus in 1914 and again in 1918 (5). This suggestion inspired and coloured the later investigations upon influenza, at any rate so far as Germany was concerned.

v. Angerer (6) found by dark-ground illumination "minute particles" in glucose-broth cultures of the filtered blood of rats inoculated with influenza sputum. He made no attempt to demonstrate that these particles were living or capable of producing disease.

Leschke (7) filtered lung-juice or sputum of cases of influenza, and in the incubated filtrate found similar particles in active Brownian movement. The filtrate of sputum inhaled in a spray by the author and several others produced no effect. The experiment was, however, repeated in September, 1918, upon an unstated number of persons and these were all attacked with influenza, but so also were two other persons in contact who had not been sprayed. This author also makes no attempt to show that he is dealing with a living virus, while his experimental transmission of the disease was obviously carried out at a time when influenza was rife among his subjects.

Selter (8) filtered the pooled throat washings of five cases of influenza, and he himself and his assistant inhaled the sprayed filtrate. Next morning he had a "cold," without fever, and a headache. The symptoms were gone in the evening. His assistant next day complained of headache and feeling tired. On the second day she (she was a female assistant) woke with a headache and temperature 37·6° C. In the second night she shivered and sweated. Fejes (9), without foundation, describes this patient as suffering from "several days fever." Thus this author gives no evidence of a living microbe in his material, neither does he reproduce a disease which has any resemblance to influenza.

Fejes himself found the same Brownian particles in ascitic broth cultures of filtered influenza sputum. He carried out inoculations upon four apes, giving each an unstated quantity, and at the same time four other apes were injected as controls with heated filtrate. These remained alive. The first four apes died in from nine to twenty-four days and, at the autopsies, revealed a picture of "hæmorrhagic sepsis." Hæmorrhages, large and small, were found in all parts of the body. No mention is made of clinical symptoms, microscopic or bacteriological examinations. It is remarked that the filtrates were only used when sterile, thus it may be assumed that the filters used were sometimes defective. This author also produces no evidence that his filtrates contained living organisms of any sort, neither does he appear to have attempted to exclude ordinary microbes as the cause of the "hæmorrhagic sepsis."

Prell (10) did little more than find granules in sections of influenzal lungs stained with iron-hæmotoxylin. These granules he called *Ænigmoplasma influenzæ* and claimed that they were the cause of influenza.

In France the filter-passing concept was supported by Nicolle and Lebailly (11). Seven experiments were carried out upon men and monkeys and in three "positive" results were obtained. The first need not be discussed since it relates to the inoculation of whole influenza sputum under the conjunctiva of a monkey with the production of fatigue, diarrhœa, wasting and depression. Some more of the sputum was diluted, filtered through a Chamberland candle and inoculated subcutaneously into a man: "he fell ill on the same day as the monkey; he presented the classical symptoms of influenza, sudden onset, headache, general depression, oculo-nasal catarrh." The temperature rose to 102° F. In another similar experiment upon man, "the symptoms were those of a slight influenza." The other experiments were negative.

As in the German work, there is no evidence in this of a living virus. Passage experiments were either negative or not attempted. The authors merely demonstrated the presence of a toxic substance in sputum. The same substance might also be present in non-influenzal sputum.

In England, Rose Bradford, Bashford and Wilson (12) claimed to have cultivated a filter-passing organism from a number of diseases, including influenza. Owing, however, to the criticisms of Arkwright (13) these claims appear to have been withdrawn.

Gibson, Bowman and Connor (14), also carried out a large series of experiments with the same objects, but owing to the unfortunate death of one of the workers, the research was left unfinished. They inoculated filtered influenza sputum into Noguchi's medium and observed the growth of a cloud above the fragment of kidney. On prolonged staining this cloud was found to consist of minute "coccoid bodies." These bodies upon being transferred to other Noguchi tubes developed again and were apparently propagated indefinitely. These cultures, inoculated into sundry animals, produced dilatation of the lung capillaries with hæmorrhages of varying extent and exudates into the alveoli or bronchioles. The coccoid bodies were recovered in culture from the kidneys of these animals, and these cultures reproduced the disease. The kidneys of normal animals failed to grow these coccoid bodies.

These experiments were published so shortly that it is a little difficult to criticise them. It is not clear in what way the coccoid bodies differ from the similar bodies which, in spite of their statement to the contrary, are to be observed in uninoculated Noguchi tubes or, indeed, in many albuminous mixtures. They are clearly the same bodies as the "minute particles" of the Germans. Angerer himself (6) admits that hæmoglobin is the best medium for their development. Olsen (15) also was able to manufacture them at 37° C. or 56° C. Again it is not quite clear whether the descriptions of the lesions mentioned above apply to the passage animals or to other animals inoculated with other materials in other experiments. It is a general composite description of the usual results of their work. The only definite statements with regard to the passage experiments are "Its lungs showed some hæmorrhagic areas," and "although the lungs of the animal did not show any typical signs of a marked infection."

The composite picture itself, illustrated by photographs, appears to be based largely upon the results obtained with monkeys, and it is notorious that inflammatory lesions in the lungs of these animals in captivity occur spontaneously.

It is thus seen that as the matter is left the experiments are not conclusive.

The filter-passing work upon influenza has been often repeated but with negative results, and these have usually remained unpublished. However, Lister and Taylor (16) published negative results. Eleven men, who had never had influenza, were removed 500 miles to an island where influenza had not occurred. Nasal washings and sputum from early cases of influenza, both filtered and unfiltered, were introduced into the noses of these men and also into monkeys. In every case the filtered material produced no effect, while two men who received the unfiltered washings developed typical influenza. At the time of the experiment the epidemic had passed.

Wahl, White, and Lyall (17) made extracts from influenzal lungs and sprayed this material, filtered, into the noses and nasopharynges of two volunteers. They remained well.

During the height of the epidemic in November, 1918, one of us (J. McI.) attempted to confirm the existence of filter-passing organisms in influenza.

(1) The filtered nasal washings from two early uncomplicated cases of influenza were inoculated into two *Macacus rhesus* monkeys, four rabbits and two guinea-pigs; 0·1 c.c. was given subconjunctivally and 5 c.c. subcutaneously. There was no effect.

(2) The filtered throat washings from three acute early cases were inoculated in the same way (but 8·0 c.c. subcutaneously) into a *Macacus rhesus* and a *Hussar* monkey. There was no effect.

(3) The filtered lung-juice from necropsies on three fatal cases was inoculated, as in the first series, into a *Macacus rhesus*, a dog-faced monkey, and two rabbits. The dog-faced monkey showed a result as follows: "This animal,

ten days later, developed a running at the nose and a temperature of 103·2° F., but an uninoculated monkey in the next cage had a bad cold. The animal got gradually weaker and died thirty-four days after the inoculation. A necropsy showed an accumulation of fluid in the pleural and pericardial cavities. The lungs showed no congestion, only a few consolidated areas. The spleen was slightly enlarged and showed a number of small yellow areas. Bacteriological and histological examination revealed a recent septicæmia with miliary abscesses. A passage inoculation was made into a *Macacus rhesus* without result."

We do not look upon this as a case of influenza produced in a monkey, but as a spontaneous disease of non-influenzal origin.

To sum up, the positive results of the filter-passing work upon influenza are unconvincing. The characteristic microbe adduced is indistinguishable from undoubtedly inanimate particles which are found in albuminous fluids. The attempts to demonstrate life in these consist in observing the development of cloudiness in other albuminous fluids after inoculation with the particles. Uninoculated tubes are sometimes said to be unaffected, but we ourselves, when engaged upon other work, have seen such opacities develop spontaneously in Noguchi medium. We have thought that they might be globin from the dissociation of hæmoglobin. Such a dissociation might be accelerated by ferments derived from the inoculum or from the kidney, or might be due merely to the action of heat upon this unstable substance.

Arkwright (13) in his attempts to grow the virus of trench fever and influenza in Noguchi's medium, observed these opacities in inoculated or uninoculated tubes. He had the opportunity of examining the preparations and cultures of Bradford, Bashford and Wilson, and found that the bodies described by them as the virus of influenza and of other diseases were identical to the bodies in uninoculated tubes, and, further, the cultures said by them to be pure were in fact contaminated with ordinary bacteria.

It may be pointed out that these coccoid bodies from Noguchi medium have now been described as the cause of common colds, influenza, trench fever and eleven other diseases.

Under the circumstances it is impossible to accept them as bearing upon the influenza problem.

With regard to the experimental transmission of the disease by means of filtrates, those relating to man are unsatisfactory in that the subjects were not isolated or that the illness produced was not influenza. It cannot be said that the lesions in animals were not those of influenza because the effect of this virus upon animals is unknown.

THE RELATION OF *B. INFLUENZÆ* TO INFLUENZA.

As already indicated, reports on the *incidence of B. influenzæ* in influenza have been highly contradictory. It is clear that in the recent epidemic, complicating infections with pyogenic cocci were very much more numerous than in Pfeiffer's day, and so much more difficult was it to isolate the minute colonies of B. *influenzæ* on blood-agar. The reports from the earlier stages of the epidemic, therefore, show a general inability to demonstrate the bacillus in more than a small number of cases.

The situation was, however, entirely altered by the re-discovery of the importance of altered as opposed to unaltered blood for the growth of the bacillus. These media have a marked selective action and enormously increase the ease with which B. *influenzæ* may be recovered. Attention should, therefore, only be directed to the work of those authors who used one of these selective media.

In the sputum of uncomplicated cases of influenza Matthews (18) found B. *influenzæ* constantly (12 cases), Eyre and Lowe (19) in 12 out of 14, Fildes, Baker and Thompson (20) in 12 out of 15, and McIntosh (21), in an acute epidemic among nurses, in 8 out of 12. Martin (22) investigated a small outbreak in France and found B. *influenzæ* in 76 per cent. More recently Schorer (23) in 81 cases found the bacillus in 91·5 per cent. On the other hand, Little, Garofalo and Williams (24) failed to find B. *influenzæ* in the exudates from the upper air-passages and used this as an argument against the influenzal nature of the epidemic. The negative results, however, are to be explained on the grounds that they used unsuitable medium (legumen-serum-agar). The numerous negative findings among German workers—Gruber, Kolle, Benda, Friedemann, Kruse, etc.—as reported by Friedberger and Konitzer (25) are probably due to the same cause.

In the sputum of cases complicated with pneumonia the presence of other bacteria tends to dilute or cause a disappearance of B. *influenzæ* to some extent, especially in the epidemic period, but Hammond, Rolland and Shore (26) investigated an epidemic of "purulent bronchitis" during the winter of 1916–17 before the main epidemic started. In a series of 20 cases the bacillus was found in 18 by microscopic and cultural examinations. They therefore considered that B. *influenzæ* was the cause of the epidemic on the grounds that the organism was almost constantly present in the sputum and pus of affected bronchioles, in some cases uncontaminated by any other organism. Shortly after this Abrahams, Hallows, Eyre and French (27) grew B. *influenzæ* from 7 out of 8 cases of purulent bronchitis.

At the height of the epidemic period Eyre and Lowe (19) isolated this bacillus in 12 out of 14 sputa, Fildes, Baker and Thompson (20) in 45 out of 106 cases, McIntosh (21) in 42 out of 69, while Messerschmidt, Hundeshagen and Scheer (28) demonstrated Pfeiffer's bacillus in 48·9 per cent. of the June–July epidemic and in 90 per cent. of the September epidemic. Pfeiffer (29) himself states that in 217 sputa sent with the clinical diagnosis of acute influenza, the bacillus was present in 51 per cent. Dick and Murray (30) found it in 63·2 per cent., Park (31) in 80 per cent., Medalia (32) in a large series of 2279 cases in 76·8 per cent., and Schorer (23) in 93·8 per cent. of

260 cases. The variation in these figures depends very largely upon the number of examinations made in each case and the quality of the specimens of sputum forwarded for routine examination.

In the blood before death *B. influenzæ* is only present on rare occasions. Fleming (33) and Medalia (32) found it a few times, but most workers gave uniformly negative results (Fildes, Baker and Thompson [20], McIntosh [21] and Dick and Murray [30]). Wilson and Steer (34) found the bacillus twelve times in the heart-blood post mortem and similar findings have been recorded by others, but in such cases it is always associated with other bacteria. Isolated records are found of the bacillus in empyemata, pericarditis, meningitis, etc.

At autopsies in the beginning of the influenza epidemic almost the only organisms of importance which were isolated from the lung lesions were streptococci and pneumococci. Much time was spent without obtaining any evidence of the presence of *B. influenzæ.* The introduction of selective media, however, resulted in the demonstration of the bacillus in a large number of cases, as may be seen from the figures given below. Thus, Fildes, Baker and Thomson (20), as also Patterson, Little and Williams (35), record its presence in almost all post-mortem material. Muir and Wilson (36) were successful in 30 fatal cases, and remarked that *B. influenzæ* at least played a very important part in the production of the lesions. Lister (37) isolated *B. influenzæ* 53 times from the lungs of 56 fatal cases in South Africa. Olsen (15) found the bacillus in 75 per cent. of the lungs of 220 post mortems, Tytler, Janes and Dobbin (38) in 60 out of 67 cases, Keegan (39) in 82 per cent., Leichtentritt (40) in 60 per cent. McIntosh in an unpublished series found the bacillus in 40 out of 50 cases, and in a second series in 94 per cent. The negative findings have been recorded particularly by a number of German pathologists whose names have already been noted.

Histological examination has shown that *B. influenzæ* occurs in close relationship to the pathological lesions of the disease. It is found throughout the whole respiratory tract from the tonsils to the alveoli of the lungs. In uncomplicated cases it is present in a purer state the lower the examination is made. In films from the lower end of the trachea or of the pus which may be expressed from the bronchioles the bacillus is often found in a pure state and in large numbers and characteristically enclosed within leucocytes (Dietrich (41), Wolbach (42) and personal observations). Beyond the lymphatic tissues of the trachea and lungs, there is, as a rule, no general invasion of the tissues such as may be the case with streptococci and pneumococci in fatal cases. From time to time, however, *B. influenzæ* is to be found in the heart-blood and in empyema pus, while the serous exudates in the neighbourhood of inflamed tracheal and bronchial glands frequently contain the bacillus.

B. influenzæ has been demonstrated also in the respiratory tract of a variety of other conditions such as measles, whooping-cough, phthisis, etc., but at present the exact frequence is difficult to estimate as the figures given by different workers vary greatly. In such conditions as measles in which the highest findings have been obtained the differentiation from *B. pertussis* (Bordet) must complicate matters. In trachoma, hæmophilic bacilli indistinguishable from *B. influenzæ* have been found in as high percentage as 50, but in this case it is quite possible that *B. influenzæ* plays an important *rôle* since personal observations have shown that this bacillus is not an uncommon cause of acute purulent conjunctivitis. But estimates based on patients in hospital wards, and especially during an epidemic, tend to be fallacious, owing to the rapidity with which one carrier of the bacillus can infect the remainder of the ward. The same, of course, is true with any of the microbes to be found in the respiratory tract. In the case of hæmolytic streptococci Cole and MacCullum (43) found that in the measles ward the incidence was 56·5 per cent. although on admission the incidence was only 11·4 per cent.

The extent to which *B. influenzæ* is normally present in the nasopharynx of healthy individuals is difficult to estimate, as most of the figures available are based on observations made in an epidemic period. It will, therefore, be necessary to wait for some time until accurate and reliable figures can be obtained. Fildes, Baker and Thompson (20) in November, 1918, examined 71 apparently healthy men and obtained 15 positive cultures—21·1 per cent. Later, Fildes (unpublished observations) examined 76 boys on entering a training establishment and found 18 positive. In February to March, 1919, he made cultures from the throats of 177 healthy men in a barracks and obtained 64 per cent. positive. In a training school for girls, Wadsworth (44) was able to find *B. influenzæ* in only 3 of 461 inmates. Lord, Scott and Nye (45) found bacilli in the throats of 76 per cent. of 34 students; Opie, Freeman, Blake, Small and Rivers (46) in 35 per cent. of soldiers in camp. Pritchett and Stillman (47) found that 43 per cent. of normal individuals in the personnel of a large hospital carried the bacillus.

To sum up, therefore, the question of the incidence of *B. influenzæ* in influenza, there can be no doubt that with a satisfactory technique it can be demonstrated in such a large number of cases that it qualifies for being considered of ætiological importance so far as Koch's first criterion is concerned. Not only is it present in a large proportion of cases but it has a close relationship to the lesions of the disease. It is true that it is also found in a number of conditions which are not influenza and in a large number of normal persons, at any rate during epidemic periods, but as has been pointed out above, these facts do not in themselves in any way militate against it being the cause of influenza.

The association of B. influenzæ with other bacteria.—We have already mentioned that in the pneumonias which complicate influenza *B. influenzæ* is apt to be overgrown by a number of other bacteria. These infections with streptococci and pneumococci were the outstanding feature of the change from a relatively benign disease which was observed during the height of the epidemic. The pathological picture changed to one of an acute septic broncho-pneumonia and in the lesions were found innumerable cocci and but few *B. influenzæ*. From time to time these septic microbes were described as the cause of influenza, but mostly they were recognised as secondary infections in persons intoxicated with the influenza virus.

It was characteristic that the nature of the infecting organism was different in different localities, or even changed in the same locality.

At one period they might be pneumococci, at another hæmolytic streptococci, and at yet another Gram-negative cocci. In this country streptococci were more frequent than pneumococci, although in America the opposite seemed to be more often the case.

Thus Fildes, Baker and Thompson (20) found hæmolytic streptococci in the majority of their post-mortems, Opie, Freeman, Blake, Small and Rivers (46) found that the incidence varied from 4 to 14 per cent. according to the type of case. Cole and MacCallum (43) also found hæmolytic streptococci in a large number of cases in an epidemic of pneumonia, and frequently in association with *B. influenzæ*.

Pneumococci were found to be present by Opie and his co-workers in from 53·6 to 69·8 per cent., and by Small and Stangl (48) in 72·7 to 84·4 per cent. McIntosh (unpublished) found pneumococci in about 30 per cent. and streptococci in 30 to 37 per cent., about one-half of which were of the hæmolytic variety.

Staphylococci were not so infrequent as would be imagined; in some instances they appeared to take on epidemic form, and it was suggested (Patrick [49]) that they were of some ætiological importance in epidemics of broncho-pneumonia. Fletcher (50) demonstrated the presence of meningococci in large numbers in association with *B. influenzæ* in the lungs of eleven fatal influenzal broncho-pneumonia cases.

From time to time other organisms such as the pneumo-bacillus, pleomorphic streptococci, *B. proteus*, etc. were found in considerable numbers.

Thus an association or a superimposed infection of various other bacteria, which resulted in an increased virulence of the disease, was a feature of the recent epidemic.

Attempts to demonstrate directly a *pathogenicity of B. influenzæ* to man have been almost uniformly unsuccessful. Lister and Taylor (16) introduced living suspensions of this bacillus and of two varieties of cocci into the noses and mouths of 9 men, and only 1 of those who received *B. influenzæ* fell ill. The authors would not, however, ascribe this illness to influenza. Rosenau (51) treated 19 men with thirteen strains in suspension with negative results, and Wahl, White and Lyall (17) were equally unsuccessful with 5 men.

Although, therefore, there is no satisfactory evidence that *B. influenzæ* is pathogenic to man so far as the inoculation of cultures goes, nevertheless the constant occurrence of anti-bodies to this bacillus in the serum of patients makes it clear that it has a definite pathogenic action, which cannot, however, be reproduced. The presence of the anti-bodies may be taken as evidence that the bacillus is a pathogenic bacillus such as might be the cause of influenza, but not that the characteristic lesions of this disease are due to this pathogenic bacillus.

The pathogenic action of *B. influenzæ* upon animals has been studied by many. In general it may be said that the effect of suspensions of bacilli from pure slope cultures, even in massive doses, is remarkably slight on laboratory animals. Introduced into the peritoneal cavity, a certain amount of inflammation is found depending upon the strain, but the bacteria rapidly disappear, and, as a rule, no permanent effect is produced.

On the other hand, liquid cultures very often exhibit marked pathogenic action. The success of these experiments depends upon the selection of a strain with the required effect, since many have little toxicity even under these conditions and the toxicity cannot, apparently, be increased by passage.

When introduced into the peritoneal cavity of the guinea-pig a marked peritonitis may occur with œdema of the belly wall, diarrhœa and prostration. The suprarenals are red and congested, but no constant effect is observed in the lungs.

Blake and Cecil (52) by means of liquid cultures which had been passed through a series of mice and then recovered from the peritoneal cavity of monkeys, were able to produce in monkeys an infection of the upper respiratory tract which might be complicated by acute sinusitis, tracheo-bronchitis and broncho-pneumonia.

In the first series 12 monkeys were inoculated by swabbing the naso-pharynx with a swab dipped in the culture or by directly dropping the liquid culture into the nose by means of a pipette (1 c.c.). In the animals successfully inoculated there was a rise of temperature with symptoms of coryza, blinking of the eyes, rubbing of nose, etc. The infection as a rule lasted from three to five days. Complications set in in from twenty-four to forty-eight hours, and were usually marked by a cough and scanty muco-purulent secretion. Complications which developed were 5 cases of sinusitis of the antrum of Highmore and 2 of broncho-pneumonia. Necropsies of the two latter showed extensive hæmorrhages, peribronchial areas of consolidation with exudate of leucocytes, mononuclear and epithelial cells, together with an infiltration and thickening of the alveolar walls. *B. influenzæ* was obtained from both lungs in pure culture.

In the second series 10 monkeys were inoculated with 1 to 5 c.c. of the culture intratracheally through the skin by means of a syringe. Of the 10 monkeys thus inoculated 2 developed tracheo-bronchitis and 7 pneumonia. Necropsies of the pneumonic monkeys showed a widespread patchy pneumonia, with extensive hæmorrhages, emphysema, œdema and slight cellular infiltration. In some of the animals the type of pneumonia was rather different in that it was much more general and there was extensive bronchitis. The authors considered that the pathological picture in these lungs was identical with that observed in influenza in man.

The cause of the toxicity of liquid cultures was shown by Parker (53, 54) to be due to the presence of a bacterial toxin. If a liquid culture of a suitable strain was filtered and injected intravenously into rabbits the animal would die in one to two hours. By preparation of animals with this toxin it was possible to produce an anti-toxin which had a certain degree of protective action against the toxin.

Similar results were obtained by Ferry and Houghton (55). Huntoon and Hannum (56) prepared an endo-toxin by grinding the desiccated bacteria and extracting with saline. This had a toxic action on mice and other animals, and, according to the authors, by whatever route it was introduced it caused acute congestion of the lungs and hæmorrhagic exudates into the pleural cavities.

McIntosh (unpublished) found that for rabbits the toxicity of the filtrates of these cultures was demonstrated in quantities of 5 to 6 c.c. or less. In some cases death followed immediately after intravenous injection, in others the animal became ill and lay in a collapsed condition, death usually occurring next day. In these animals a post-mortem examination revealed numerous subcutaneous hæmorrhages and characteristic lung changes.

In the guinea-pig the average intravenous lethal dose was 2 or 3 c.c., although with some cultures it was as low as 0·5 c.c. or less.

Perhaps the most suggestive syndrome was that observed in guinea-pigs when death occurred in four or five hours after the injection. Immediately following the injection the animals became collapsed, the hair stood on end, and they tended to crouch in a corner of the cage. About an hour later the temperature was found to be subnormal, there was a discharge of frothy mucous at the nose, and slight diarrhœa. The pathological changes observed were often quite striking, in fact it was surprising that such extensive changes could have occurred so quickly. This was particularly true of those animals which died in from twenty-four to forty-eight hours. The changes were identical with those observed in rabbits, only they were more intense. The lungs were, as a rule, voluminous and did not retract fully when the chest was opened. On the surface were to be seen slightly sunken red areas of collapse alternating with paler areas of emphysema. In some, definite hæmorrhagic areas were observed, and these at times involved a whole lobe of the lung. On cutting into the lungs, blood-stained, frothy mucus escaped in considerable amount.

Microscopically the affected areas showed a typical picture of bronchopneumonia. The bronchioles were plugged with desquamated and proliferated epithelium, together with a few leucocytes. There were hæmorrhages into some bronchioles. The bronchial walls and the peribronchial interstitial tissues were infiltrated and contained small hæmorrhages. Groups of alveoli were filled with desquamated epithelium and leucocytes. Many alveoli contained hæmorrhages and coagulated albuminous fluid.

The supra-renals were œdematous and frequently the seat of numerous hæmorrhages. The kidneys showed a varying amount of parenchymatous degeneration with actual necrosis of the epithelium in places. The intestines were usually congested and distended with gas, and in places showed subperitoneal hæmorrhages.

These lesions appeared to be characteristic of *B. influenzæ*, and were not caused by the injection of filtrates from cultures of streptococci or pneumococci.

Thus, by applying *B. influenzæ* to animals in a form which would not be permissible to man, a constant pathogenic action may be demonstrated in many strains, and the outstanding feature of the lesions produced is a broncho-pneumonia.

The pathogenic action of *B. influenzæ* in association with other bacteria has been studied. Huntoon and Hannum (56) found that the mixed toxins of *B. influenzæ* and streptococcus were more toxic than that of *B. influenzæ* alone, and that if animals were prepared with *B. influenzæ* toxin they might succumb to a spontaneous streptococcal or pneumococcal pneumonia. Yanagisawa (57) also found that the presence of *B. influenzæ* increased the pathogenicity of streptococci and pneumococci.

These results may account for the frequence of virulent secondary infections in influenza.

To sum up the evidence of pathogenicity of *B. influenzæ* to animals, it may be accepted as established that fairly constant lesions may be produced by certain cultures applied in particular ways to susceptible animals. The most characteristic lesion to be observed is a broncho-pneumonia.

The precise relationship of these experimental lesions in animals to influenza in man is difficult to define, because the true influenza syndrome is not yet clearly recognised, and the disease is very little susceptible to experimental transmission. Very extensive efforts have been made in America to transmit influenza to man experimentally (Rosenau [51]). Mixed nasal and throat washings from patients were sprayed into the noses and throats of 10 volunteers or even directly transferred on a swab; 10 c.c. of blood from influenza patients were also inoculated into 10 men; and, finally, 10 men deeply inhaled the breath and received the coughs of 10 patients. None of these men developed any illness.

On the other hand Lister and Taylor (16), using nasal washings and sputum of cases, described two positive results. They introduced this material into the throats and nasal cavities of men and monkeys, and two men out of five developed influenza, although the same material, filtered, produced no effect.

In the absence of precise knowledge, then, we may define influenza as an infective illness which is extremely difficult to transmit wilfully to another person. Its characteristic lesion is an inflammation of the respiratory tract, especially of the trachea and bronchioles in more advanced cases. Depression and intoxication are also conspicuous. Finally the disease is liable to acute outbursts during which the benign character changes, and then it becomes associated with numerous septic bacteria.

The disease produced in animals by *B. influenzæ* is also only brought about with difficulty under conditions which ensure the presence of a large amount of toxin. The bacillus itself is nearly ineffective. The disease when produced is essentially an inflammation of the respiratory tract which has a very close resemblance to that observed in many cases of influenza. The intoxication of the animals is always well marked. Lastly there is evidence that an intoxicated animal is more susceptible to the attacks of other bacteria than a normal animal, and may even spontaneously develop pneumonia.

Thus, we have a characteristic living organism which is present in the lesions of influenza and has a pathogenic action for man. Pure cultures of this bacillus are capable of producing in animals a condition which has important points in common with influenza in man, and it is only the indefinite nature of the essential lesions of influenza which causes hesitation in accepting the experimental disease in animals as the same as the natural disease in man.

If this relationship is accepted, then the criteria by which a bacterium should be judged the cause of a disease are fulfilled.

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OF

EXPERIMENTAL PATHOLOGY

THE OXYGEN CONSUMPTION OF NORMAL AND CANCEROUS MOUSE TISSUES *IN VITRO.*

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DESPITE the prolonged and exhaustive investigations made upon the growth of transplanted tumours, but little work has been done upon the metabolic processes which lie at the root of such growths. This is attributable in the main to the small amount of tissue to be dealt with when working with rats and mice, the two species whose tumours have lent themselves best to experimental investigation. As regards the gaseous metabolism of tumours, the only work we can cite is that of Cramer (1) and of Chisolm (2), who respectively studied the respiratory exchange of tumour-bearing rats and mice. Cramer was unable to detect any essential difference between the tumour rats and the control rats, either as regards the absolute amounts of oxygen and carbon dioxide involved or the respiratory quotient. Chisolm came to the same result after comparing tumour-bearing mice with normal mice.

Most recently Drew (3) has published observations made in this laboratory upon the rate at which normal and cancerous tissues of the mouse reduce a dilute solution of methylene blue. He has found large differences between the different tissues and lesser differences between the various tumour tissues, whilst the tumour tissues were all much less powerful in reducing the methylene blue solution. We shall have occasion to refer to these findings more particularly later.

In attempting to assess the oxygen consumption of tumour tissue one is severely handicapped by several factors, the more serious being the restriction of choice of animal, and the defective circulation in tumours, with its concomitant load of dead tissue taking next to no part in the gaseous exchange but upsetting the calculation of quantities severely. The method so successfully applied by Barcroft and his co-workers in estimating the oxygen consumption of various organs from the different content of oxygen in afferent and efferent blood is not available. The blood-vessels of mice and rats are too small and delicate to allow of this manipulation. The successful implantation of a suitable tumour into the kidney of such an animal as the rabbit might supply the necessary conditions for an analysis on those lines, but no such tumour was available. Under these circumstances we were impelled to conduct our investigations in a less ideal condition, and to test the oxygen consumption of the normal and cancerous tissue *in vitro*.

It has long been known that the tissues of an animal are capable of absorbing oxygen and of giving off carbon dioxide for a considerable time after the death of the animal. More recently Battelli and Stern (4), in a series of papers, have published their findings on post-mortem respiration. In general their method of procedure was to suspend a fine emulsion of the tissue in blood and expose it to an atmosphere of oxygen at 38° C. The readings they obtained were very high; pigeon muscle in ox blood absorbed oxygen at the rate of 66 c.c. per kilogram minute; dog's liver in an emulsion of dog's red blood cells absorbed 60 c.c. per kilogram minute. Harden and Maclean (5), in an extensive series of experiments, were unable to confirm these high readings, and the figures they obtained showed an oxygen consumption which was only a tenth or a twelfth of what Battelli and Stern obtained. An emulsion of dog's liver suspended in ox blood, for example, was found to consume 3·8 to 6·6 c.c. per kilogram minute, figures differing radically from those of Battelli and Stern.

Before we came to the estimation of the oxygen consumed by different transplanted mouse tumours, it was necessary to obtain figures for various normal organs of the mouse, as it was only to be expected that the mouse with its very active metabolism would yield figures very different from those found by Harden and Maclean for larger animals. The only observation made on mouse tissue was that of Usui (6), who estimated the oxygen consumption of the left lobe of the liver in a 10 per cent. suspension of ox blood corpuscles and found figures varying from 4 to 9 c.c. per kilogram minute. Usui differed from his predecessors in not using an emulsion of tissue but in dealing with the lobe of liver in its entirety, but this obviously had the drawback of the central portion not receiving an adequate supply of oxygen.

The method employed in making the present series of determinations has been as follows:—With the exception of two experiments done with mouse blood, rabbit blood has been used throughout as a source of oxygen. This has been obtained from an ear vein after thorough cleansing with ether; in this way it is an easy matter to obtain 30 c.c. or more of defibrinated blood which is often sterile, and never contains more than a few organisms which do not disturb an experiment of two or three hours' duration. Absolute sterility of the blood need not be aimed at, since the mouse tissues to be investigated usually contain a few organisms. The blood is oxygenated by shaking or rotating for five minutes in a flask. The mouse is then killed and the tissue to be tested removed and rapidly emulsified with a pair of curved scissors. With tumours, care has been taken to remove all naked-eye necrotic material. A weighed quantity of this is added to a measured quantity of the rabbit's blood in a cylindrical glass vessel of 28 mm. diameter provided with a stirring rod and covered with a layer of liquid paraffin. The quantities commonly employed have been half a gramme of tissue emulsion in 12 c.c. of blood, these quantities being varied either way with a view to attain the consumption of half the available oxygen after two or three hours' incubation. The mixture is then incubated in a water-bath at 37·5° C. A control tube containing 10 c.c. of blood without any added tissue but otherwise similarly equipped is also incubated. This tube is necessary to ascertain the rate of auto-oxidation, which has been found to vary considerably. Ten minutes' incubation is allowed for equilibrium to be established, and the first sample of blood is then withdrawn with a 1 c.c. pipette containing a little warm paraffin. The sample is next analysed in Barcroft's blood-gas apparatus, and the amount of oxygen necessary to complete saturation is estimated. This is repeated after further incubation at hourly intervals for the blood and tissue tube, but for the control tube, on account of the small differences noted, a second sample has usually been taken only after two or three hours' incubation. In all the experiments the time elapsing between the death of the mouse and the taking of the first blood sample has been between twenty and twenty-five minutes.

The course of an experiment will be seen in the following protocol where the oxygen absorption of liver emulsion is given in detail.

14/4/20.—Rabit bled at 11.15 a.m.; animal last bled twenty-two days previously. Mouse killed at 11.20 a.m., and 0·63 grm. of liver emulsion added to 13 c.c. of defibrinated rabbit's blood. As control 9 c.c. of blood was taken. Incubation started at 11.34 a.m. in water-bath at 37·5° C.

Blood with emulsion of liver:

Barcroft apparatus ¾ (K = 3·36). Temp., 14° C. Bar. Pr., 750 mm.

Time	11.45 a.m.	12.45 p.m.	1.45 p.m.	2.45 p.m.
Oxygen absorption in mm.	4·5	14·5	26·5	30

Control blood :

Barcroft apparatus ½ (K = 3·17). Temp., 14° C. Bar. Pr., 750 mm.

Time	11.50 a.m.	2.50 p.m.
Oxygen absorption in m.m.	3	5

Re-calculation of the above figures after allowance for auto-consumption gives during the first hour an oxygen consumption of 9·2 c.c. per kilogram minute; 10·0 c.c. for the second hour; and 2·3 c.c. for the third hour. The auto-consumption of the blood was exceptionally low in this experiment; more usually the figure obtained is 0·0060 volumes per cent. per minute. The highest figure found was 0·0160, but the rabbit had been bled only ten days before. There was no evidence pointing to the rate of auto-consumption affecting that of the tissue tested either way.

In Table I the rate of oxygen consumption is summarised for the different normal tissues tested, the figures being given in c.c. per kilogram minute.

TABLE I.

Organ.	First hour.	Second hour.	Third hour.	Amount of tissue in grms.
Kidney	21·5	20·1	16·1	0·42
Ditto	23·9	19·5	5·8	0·28
Ditto	18·5	15·9	—	0·44
Average	21·3	18·5	—	—
Liver	12·9	9·4	4·9	0·35
Ditto	9·2	10·0	2·3	0·63
Average	11·0	9·7	—	—
Testis	8·4	6·7	—	0·60
Ditto	4·7	4·3	6·5	0·35
Ditto	4·3	—	—	0·53
Average	5·8	5·5	—	—
Embryo	4·0	3·0	3·0	0·55
Ditto	3·0	—	—	0·66
Mamma 4 days lactating	9·8	7·0	4·4	0·56
Mamma 2 days lactating	9·3	—	—	0·42
Mamma—full term	9·1	—	—	0·33
Mamma about 3 days before full term	5·1	4·7	3·4	0·66
Mamma about 6 days before term	7·2	5·6	1·3	0·30

The mice whose tissues have been tested have all been fed in the ordinary way—that is they were living with a surplus of food at their disposal. No attempt was made to analyse the oxygen consumption of the tissues of fasting animals, although deprivation of food might very possibly affect the figures for such organs as kidney and liver. The oxygen consumption found for the various tissues is seen to differ materially, whilst the value found for any one tissue in separate experiments has been fairly close except in the case of testicular tissue, which has behaved rather erratically. The second hour readings show a distinct drop in almost all cases, whilst this drop is very marked in the third hour. In later experiments a third hour reading has more usually been omitted on account of its irregularity. Two further experiments were done on mouse kidney suspended in defibrinated mouse blood, and the values found were a third higher than in rabbit blood. The high rate of auto-oxidation of mouse blood, about six times that of rabbit, made it unsuitable for testing the more slowly oxidising tissues.

The rate of consumption by kidney emulsion in mouse blood, about 30 c.c. per kilogram minute, does not approach what might be expected for this organ under physiological conditions. Barcroft and Straub (7) in their work on secretion of urine found the kidney of the cat and rabbit usually consumes 70 to 100 c.c. per kilogram minute, and the figure for mouse kidney might well be expected to be higher. The comparatively low readings obtained when using an emulsion indicate that in the process of mincing the cells are damaged to a considerable extent. In this connection reference may be made to the deleterious influence which mechanical injury has upon the oxidative transformation of lactic acid in excised amphibian muscle as described by Fletcher and Hopkins (8). They emphasise rightly the necessity of drawing a distinction between the study of chemical processes in minced tissues and intact living tissues.

The findings for mammary tissue require special consideration, as here one is not dealing with an organ whose composition is even relatively constant, but varies considerably with the functional condition of the gland and the state of nutrition of the animal. To ascertain the degree of this variant, a large lobe of mamma was preserved for microscopic examination in each experiment, and the conditions found in each case were as follows.

The four-day lactating mamma showed about a third of the section occupied by fatty tissue. The parenchyma consisted mainly of somewhat dilated acini lined by a layer of cubical epithelium. The two-day lactating mamma contained very little fatty tissue, and the field showed a larger amount of glandular epithelium, whilst the acini were not so dilated. The mamma of the mouse taken at full term showed a large amount of fat occupying rather more than half the section, whilst the acini were more contracted. The mammæ from the eighteenth day of pregnancy and from the fifteenth consisted of equal parts of fatty tissue and parenchyma; the acini were very dilated, rendering it difficult to estimate the amount of epithelium present, but it was approximately equal to that in the full-term mamma. Comparison of these histological findings with the respective rate of oxygen consumption found *in vitro* fails to establish a parallel in the sense of a direct relationship between the amount of oxygen consumed and the amount of parenchyma present. So far as any conclusion can be drawn from the limited number of observations, it is that the oxygen consumed is parallel to the functional activity of the gland. In the first three cases milk was being secreted in visible quantities, and the rate of oxygen consumption was appreciably higher than it was in the other cases where no milk was seen. The large differences found in the oxygen consumption of kidney, liver and embryo tissues make it rather difficult to believe that one is dealing with the true basal metabolism of these tissues. A part of the difference might, in the case of embryo tissue, be attributed to its high water content, 85 per cent. for embryo tissue, against 77 for kidney and 73 for liver; but allowance for this factor still leaves an astonishing discrepancy. In the embryo the quantity of tissue functioning in the sense of doing work is small, and this again suggests that the much higher reading for kidney and liver tissues is due to a partial continuance of function for an hour or two under the conditions of the experiment. Still, as Warburg (9) has pointed out, there may be a high oxygen consumption with minimal expenditure of energy in performing work, as in the case of the isolated central nervous system of the frog, or in the nucleated red blood-cells of birds. He suggests (on page 259) that mechanical work may be necessary to retain the complicated cell structures inviolate.

The experiments on tumours have been carried out in the same way as for normal tissues. In preparing an emulsion of tumour tissue great care has been taken to remove necrotic areas, but histological control has shown the impossibility of eliminating entirely the dead tissue, and at best one can only aim at reducing it to a minimum which will be fairly constant in successive experiments. Seven transplanted mouse tumours have been tested, including six carcinomata of different rates of growth and a rapidly growing sarcoma, and the figures obtained are presented in tabular form in Table II.

TABLE II.

Tumour.	First hour.	Second hour.	Third hour.	Amount of tissue in grammes.
Carcinoma 206	9·7	5·7	—	0·44
Ditto	5·5	2·6	—	0·65
Sarcoma 37	5·5	6·2	—	0·56
Ditto	9·3	7·4	—	0·60
Ditto	5·4	4·9	0·8	0·57
Average	6·7	6·2	—	—
Carcinoma 63	5·7	4·9	—	0·60
Ditto	6·7	0·0	3·8	0·48
Ditto	6·1	—	—	0·46
Average	6·2	—	—	—
Carcinoma 199	5·3	3·5	—	0·67
Ditto	4·0	—	—	0·69

Tumour.	First hour.	Second hour.	Third hour.	Amount of tissue in grammes.
Carcinoma 113	3·8	4·4	3·4	0·63
Ditto	4·8	0·6	3·2	0·60
Carcinoma 72	5·4	1·2	—	0·48
Ditto	6·4	1·4	—	0·60
Carcinoma 155	3·5	1·8	—	0·80
Ditto	8·8	2·1	3·0	0·44

From this table it will be seen that there is, in general, about the same drop in the rate for the second hour as was previously noted for the normal tissues. The consumption during the first hour further shows considerable differences for the same tumour strain. These wide differences in the same tumour strain we believe to be actual, and not accidental or attributable to errors of technique. Sarcoma 37, a rapidly growing spindle-celled tumour, for example, shows a wide variation between 5·4 c.c. and 9·3. The most consistent readings are those with carcinoma 63, a rapidly growing carcinoma which has been growing very uniformly for several years. Carcinoma 206 can at times grow with extreme rapidity, but it behaves very erratically. The high reading of 9·7 c.c. was obtained from such a rapidly growing specimen.

The very discrepant readings obtained with carcinoma 155 seem also to coincide with a differing rate of growth. This is the slowest growing tumour tested, but the specimen which gave the high reading grew exceptionally fast, although still very slowly when compared with strain 63.

In Fig. 1 the rates of oxygen consumption have been plotted both for the normal and neoplastic tissues, the average figures obtained for the first hour being expressed in terms of 5 mm. height, equalling 1 c.c. per kilogram minute. When graphically represented in this way, it can be readily seen how slight are the variations in the rates for neoplastic tissue. If a horizontal be drawn through the top of the column representing the mammæ of pregnant mice, it will be seen how the tumours group themselves about this line. Whether the tissue histogenetically related to a tumour determines approximately the rate of oxygen consumption of the latter could only be settled by comparison with transplanted tumours of other organs such as kidney or liver, material which unfortunately is not at present available.

It will be noted that the other rapidly growing tissue tested, the embryonic tissue, has a very low oxygen consumption. There is no reason to assume that this complex of tissues is more severely damaged by cutting than the other tissues, but it seems more reasonable to suppose that it works more economically than the tumour tissue.

The five columns in the figure representing carcinoma 206, sarcoma 37, and the carcinomata 63, 199 and 113, show a regular diminution. This diminution is so far consistent in that it agrees directly with the speed of naked-eye growth of the tumour strains in question. The sarcoma, as already stated, is a rapidly growing spindle-cell tumour of mammary origin, whilst the four epithelial tumours are all alveolar mammary carcinomata of different rates of growth. In all the histological structure shows an extreme degree of loss of histological differentiation. The most slowly growing tumour of this group, carcinoma 113, differs from the other three in that its cells show a unique aptitude for storing glycogen, a feature present in the primary growth and now observed in the transplanted tumour for many years.

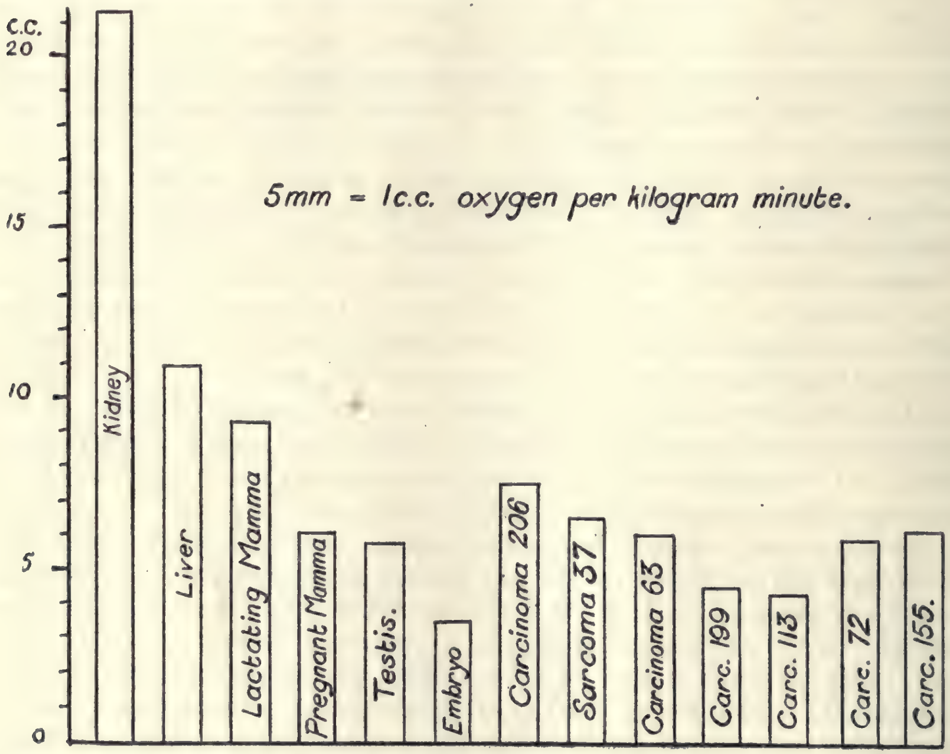

FIG. 1.—Diagrammatic representation of average rate of oxygen consumption for the first hour.

The two carcinoma strains 72 and 155 differ in two respects from the previous group. They grow very much more slowly, and show a higher degree of histological differentiation, the acinous structure of the parent mammary tissue being retained in a varying degree of perfection.

The remarkable differences which Drew found between normal tissues on the one hand, and neoplastic tissues on the other, do not appear in anything approaching the same degree in our experiments. This is attributable to the different conditions under which the tissues were tested by Drew. Whereas all our estimations have been carried out on tissues with a readily available supply of oxygen, in Drew's experiments the cells were virtually existing under anærobic conditions, and the oxygen which they used had to be split off from water, leaving the hydrogen free to reduce methylene blue.

So far as the limited differences which we have found in the rate of oxygen consumption by various transplanted tumours allows us, the following conclusions will be tentatively drawn :

That the rate of oxygen consumption rises with increasing speed of growth, and further that more oxygen is used by tumours which are more highly differentiated histologically.

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ON GLANDULAR ADIPOSE TISSUE, AND ITS RELATION TO OTHER ENDOCRINE ORGANS AND TO THE VITAMINE PROBLEM.

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ON THE OCCURRENCE AND STRUCTURE OF GLANDULAR ADIPOSE TISSUE.

THE adipose tissue in most species of animals, including man, is apparently of a uniform character in different sites of the body to the naked eye and when examined by the ordinary histological methods, namely in frozen sections stained with sudan, or in paraffin sections stained with osmic acid either directly or after previous bichromate fixation. It will be pointed out presently that this uniform appearance is only superficial and that sometimes distinct differences can be noticed. In some species of animals, however, such as the white rat and the tame mouse, for instance, two distinct types of adipose tissue can always be recognised even by the naked eye. In addition to the ordinary adipose tissue with which everybody is familiar there occurs in these species a second gland-like type of tissue, the cells of which are filled with globules giving all the typical histo-chemical reactions for fat.

It can be seen in various sites in well-defined reddish-brown masses which contrast strongly with the yellowish-white fat of the ordinary adipose tissue. The three sites where it is most abundant are—

(1) On the back between the scapulæ with extensions between the muscles. The tissue lies here in two oval, definitely circumscribed, gland-like masses, each about 1 cm. long. Smaller masses may be found also in the axilla and in the neck (Fig. 1, Plate I).

(2) In the thorax in front of the vertebral column. It appears there as a narrow brown band stretching through the thorax along the thoracic aorta (Fig. 3, Plate I).

(3) In the abdomen between the kidneys and the aorta. It lies on either side of the renal vessels, and extends both above the kidney, enclosing the adrenal, and below the kidney, enclosing (in female animals) the ovary (Fig. 4, Plate II).

Our observations have been made mainly on the perirenal cholesterin fat

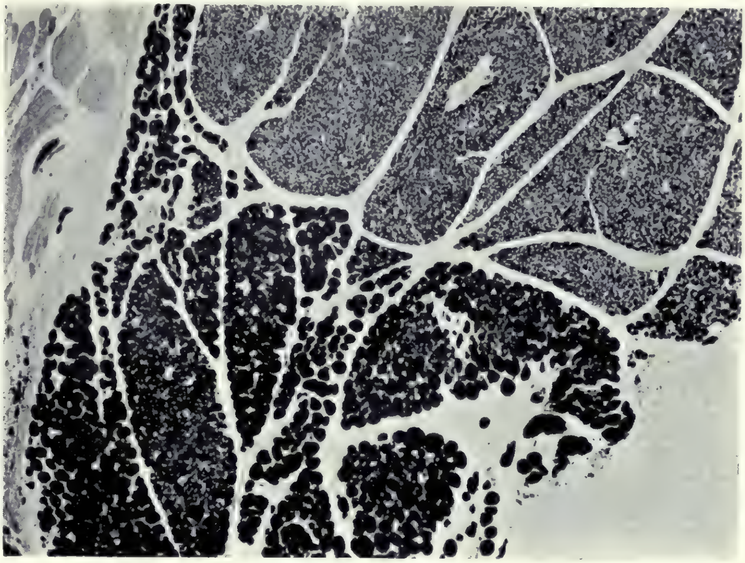

FIG. 1.

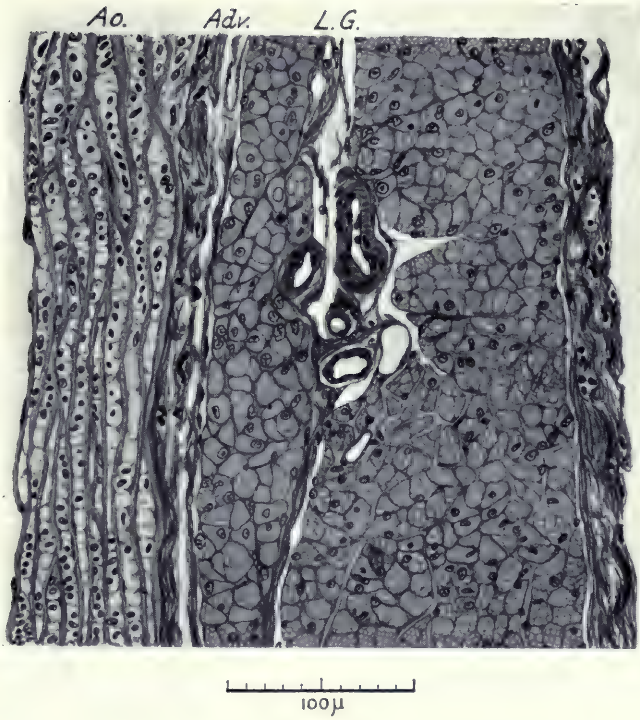

FIG. 3.

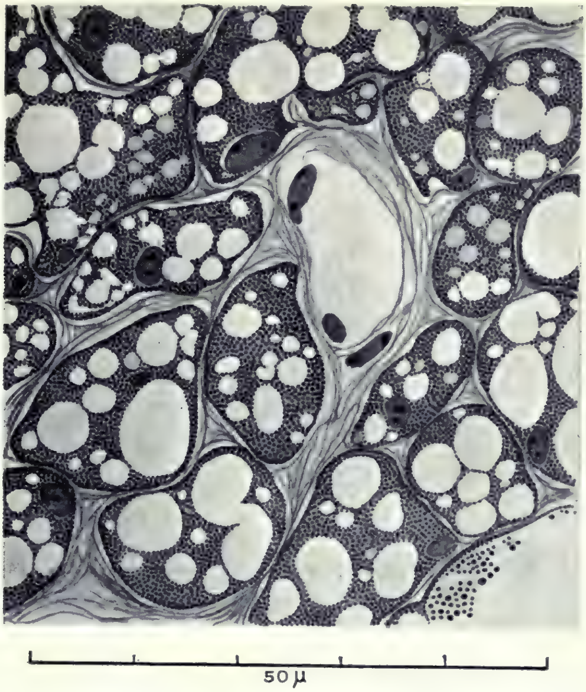

FIG. 4.

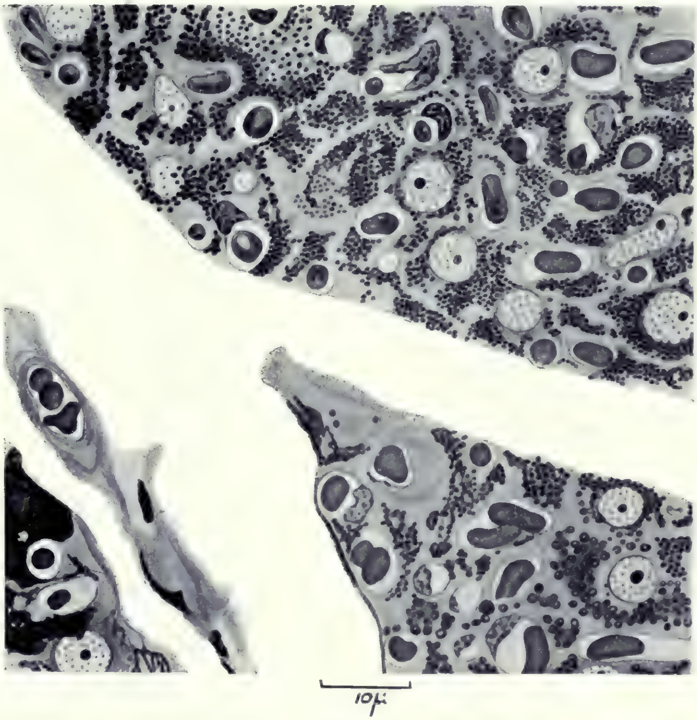

FIG. 5.

tissue and the following descriptions refer more particularly to it. Frozen sections show that the cells of this tissue contain globules which stain deeply with sudan and osmic acid. In some parts of this tissue the cells may contain only one large globule, which squeezes the nucleus to one side as in ordinary adipose tissue; in other parts, however, the tissue is made up of cells containing numerous small globules which are grouped around a central nucleus. These cells are polygonal, not round, and appear to be richer in protoplasm than the round cells of the type found in ordinary adipose tissue. This is especially well shown in paraffin sections fixed in formol or in Zenker's mixture, in which all the fat and lipoids have been dissolved out but the protoplasm has remained. In such sections the reticulated appearance of the polygonal cells with a central nucleus forms a striking contrast with the empty, oval cells of ordinary fat with the nucleus pressed against one side. The cells are arranged in distinct lobules which are richly vascularised. In the rat, in fact, this gland-like adipose tissue resembles structurally the hibernating gland as described by Carlier (1) in the hedgehog. The clearest histological picture of this tissue with the globules *in sitû* is obtained by treating the tissue with osmic acid after a previous application of potassium bichromate. The method used by us has been that recommended by Schridde for the demonstration of Altmann's granules, viz. fixing the tissue in formol-Müller's fluid for one day and, after transferring it first to Müller's fluid for two days, then to osmic acid 2 per cent. for two days, embedding it in paraffin. If such sections are examined without further staining one obtains the picture illustrated in Fig. 1. When examined in polarised light under crossed Nicols in frozen sections many of these globules show very distinct double refraction, which disappears on gently warming the slide and reappears on cooling. This anisotropic fatty material is mixed with isotropic fatty material—in other words, the globules contain a mixture of true fats and lipoids. From its histo-chemical reactions it appears that the anisotropic material consists of a mixture of several lipoid substances, since the bichromate-osmic acid treatment does not stain all the lipoids present in the tissue. We know from previous observations (2) that this histochemical reaction enables us to differentiate between different lipoids. It gives positive results with true fats and with some lipoids, but negative results with other lipoids.

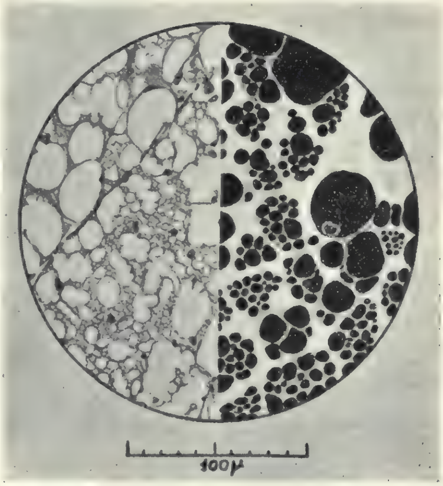

FIG 2.—(For description *vide* p. 196.)

Of the lipoids present in these globules one group can be readily identified as belonging to the cholesterol group. For if some of this tissue is dried, suspended in warm chloroform and the solution tested by the Liebermann-Burkhardt test by the addition of 10–15 drops of acetic anhydride and one or two drops of concentrated sulphuric acid, the typical greenish-blue colour develops after an interval of a few minutes.

It must be emphasised that the mere presence of anisotropic lipoid is not one of the features which distinguish this glandular type of adipose tissue from ordinary adipose tissue. The latter, too, contains anisotropic lipoids, of which one can be identified as belonging to the cholesterin group by means of the Liebermann-Burkhardt test. Nor is it possible to predict from the morphological appearance of a fat cell whether anisotropic material is present or not. It occurs not only in the polygonal cells with a central nucleus and several globules but also in the large round cells with only one large globule, although in the latter there is a preponderance of isotropic true fat. Whether all the different lipoids present in the glandular type of adipose tissue are also present in ordinary adipose tissue cannot at present be decided.

In the guinea-pig, the rabbit and in man, the fat in the immediate neighbourhood of the kidney has been studied in the same way. To the naked eye the adipose tissue in this region does not offer any obvious differences from that of ordinary adipose tissue. In man the so-called perinephric fat within the perinephric fascia sometimes feels firmer and more lobulated than the ordinary fat, but the difference is slight and is not always noticeable. Paraffin sections prepared by Schridde's method, or frozen sections stained with sudan, may show, as a rule, no difference so far as the purely morphological picture is concerned from ordinary adipose tissue, *i. e.* the cells contain one large fat-globule. Sometimes, however, one may find the same appearance as that found constantly in the rat and the mouse—polygonal cells filled with a number of small fat-globules grouped around a central nucleus. Fig 2, which is drawn from a Zenker and a Schridde preparation of human perinephric fat from a child, illustrates this point. The same appearance has been found occasionally in adults.

Although in animals other than the tame mouse and the white rat our observations have been made mainly on the perinephric tissue, the glandular adipose tissue is not restricted to that region. In the guinea-pig, for instance, it occurs in considerable masses in the neck on either side of the trachea. surrounding the glandular structures situated there (thyroid and lymphoid nodules). Its distribution in the human subject will be dealt with below.

The proportion between double refracting material and ordinary isotropic fat is very variable. The perirenal adipose tissue of the mouse and the rat is rich in anisotropic material in proportion to the amount isotropic fat present, In the guinea-pig, rabbit, and man, the isotropic material preponderated in most of the specimens of perinephric fat which have been examined.

The deposition in the form of numerous small globules is probably partly due to the fact that the cells are rich in protoplasm. But this is not the only determining factor. As long as the amount of anisotropic material is relatively large in proportion to the amount of ordinary fat, the fatty material arranges itself in the cells of this tissue in numerous small globules around a central nucleus. When the relative amount of true isotropic fat increases, as it does in well-nourished animals, these small granules as they increase in size tend to push the nucleus to one side of the cell and to coalesce into one large drop. The difference between the perirenal glandular adipose tissue of the rat and the mouse on the one hand, and of most other species on the other, is that in these latter ordinary true fat may be deposited to a variable extent in the cells of the glandular adipose tissue, so that this tissue assumes the external appearances of ordinary adipose tissue. In the mouse and the rat, on the other hand, this process does not take place to the same degree, and the tissue retains to a greater extent its distinctive appearance and structure. It follows, too, that the morphological differences—the peripheral or central position of the nucleus, the round or polygonal shape of the cells, the apparent poorness or richness in protoplasm—are not of essential significance, but probably only secondary effects resulting from changes in the size and the number of " fat "-globules present.

In the tame mouse and the white rat the glandular adipose tissue between the scapulæ and in the thorax differs somewhat from that around the kidney. Macroscopically the interscapular tissue is firmer, more definitely circumscribed, and thus conveys more the appearance of an organ or a gland. Microscopically it is more definitely arranged in lobules in which the pleoprotoplasmic, polygonal, polyglobular cells with a central nucleus predominate, especially in the thoracic fat, while under normal conditions the perinephric tissue is composed mainly of the round monoglobular cells with a peripheral nucleus. On examination in polarised light the phenomenon of double refraction is much less marked in the case of the thoracic and scapular tissue. This may be due to differences in the absolute amounts of cholesterin compounds and other double refracting lipoids present in the different sites. But it may merely be a mechanical effect resulting from the fact that in the thoracic and scapular tissue the lipoid globules are smaller and more finely dispersed than in the perirenal tissue. This latter explanation is suggested by the fact that the monoglobular round cells of the scapular fat are as strikingly double refracting as those of the perirenal tissue, while a faint double refraction is shown by the multiglobular cells. It is at present not possible to say whether these differences between the tissues in the different sites and between the two types of cells described have a functional significance.

The readiness with which the glandular adipose tissue can be recognised in the rat and mouse renders it easy to demonstate that this tissue is also functionally different from ordinary adipose tissue. For in conditions such as fasting or a restricted diet, in which the ordinary adipose tissue is greatly diminished, the glandular adipose tissue persists and shows no obvious diminution. Conversely, in very fat animals the glandular adipose tissue is not obviously increased, although it is lighter in colour and becomes surrounded by ordinary adipose tissue. Microscopically the tissue of well-nourished animals shows a mixture of cells of the monoglobular and of the polyglobular type. When the diet is restricted the monoglobular type of cell becomes transformed into the polyglobular type, so that eventually the tissue is composed of cells containing many small globules, and these cells retain their load of lipoids and fats even when the ordinary adipose tissue has entirely disappeared. The same observation has been made by Hammar (3), in his important study of the histogenesis of this special fat, which he called the "brown fat." His conclusions will be discussed in greater detail below. The difference in the behaviour of these two different types of fat under different nutritional conditions is so remarkable that some of the figures obtained by Hammar may be quoted here:

	White fat.	Brown fat.
Animal on restricted diet for 3 weeks	0·53 per cent. of body-weight.	0·60 per cent. of body-weight.
Animal on normal diet	4·5 ,, ,,	0·64 ,, ,,
	5·0 ,, ,,	0·91 ,, ,,
Animal made obese by over-feeding for 3 months	7·0 ,, ,,	1·51 ,, ,,

In other species where the glandular adipose tissue may become infiltrated to a marked degree with ordinary true fat conditions are obviously more complex, and further investigations are necessary to determine the behaviour of this tissue under different nutritional conditions.

ON THE HISTOGENESIS OF THE GLANDULAR ADIPOSE TISSUE.

If it is true that the glandular adipose tissue is a functionally distinct type of adipose tissue one would expect to find that it should also be histogenetically distinct from ordinary adipose tissue. That expectation is fulfilled. A study of the extensive literature on the development of adipose tissue shows that the embryos of all mammalian species that have been studied (rabbit, guinea-pig, cat, dog, calf, pig, squirrel, rat, mouse, marmot, hedgehog, man) show a form of adipose tissue the cells of which are arranged in definite lobules, are relatively rich in protoplasm, and contain a number of fat-granules grouped round a central nucleus. This "gland-like" fat tissue, as it has been called, may persist for some time after birth, and in the case of infants has been described and figured by Batty Shaw (4) as occurring in the subpleural tissue, in the axilla and around the kidneys. In the human subject the accumulation of this tissue which is situated in the neck has attained the dignity of a separate anatomical organ, and has been described by Hatai (5), and later, in greater detail, by Bonnot (6), under the name "the interscapular gland."

According to Bonnot its situation is on the shoulder and side of the neck between the upper border of the scapula and the clavicle, running forward, towards the trachea, enclosing the large blood-vessels, while a large process extends upwards to the mastoid process. According to the same observer. "It is pink in colour and lobulated, presenting much the appearance of a fresh pancreas," and being macroscopically recognisable even in the adult subject. The tissue undergoes hypertrophy under conditions of deficient thyroid secretion. In human cretins circumscribed swellings of "fatty" tissue have frequently been noticed to occur in the axilla and in the neck. This fact was first observed by T. B. Curling (7), who described it as "fat of a loose lobulas structure," in 1850, and his account was confirmed by Hilton Fagge (8) in 1871. Recently Shattock (9) has pointed out that this fat is not composed of ordinary adipose tissue, but is a hypertrophy of the gland-like lobulated adipose tissue which occurs normally in this region in infants. This hypertrophy suggests the existence of a functional relationship between the activity of this glandular adipose tissue and that of the thyroid. There is also evidence of such a relationship between the adrenal glands and this tissue. This will be discussed in greater detail in the last part of this paper.

In most species the characteristic histological appearance of the cells of this tissue disappears soon after birth, and the majority of authors state that it is transformed into the ordinary type of adipose tissue. In some species, however (white rat, tame mouse, and the hibernating animals), it retains its characteristic structure.

A good deal of discussion has centred around the morphological significance of this embryonic gland-like fat tissue, and the only point on which all authors are agreed is the assumption, now disproved by the observations recorded in this paper, that the fat contained therein consists of ordinary true fat and is essentially the same as that of fully-developed adipose tissue. It has been held by some authors that the adipose tissue of the adult animal is a tissue *sui generis*, and that all the cells of the adipose tissue of the adult animal are derived from this gland-like accumulation of fat tissue found in embryos, which has therefore been called "the primary fat organ." Another school of morphologists holds that adipose tissue is formed simply by the deposition of fat in connective-tissue cells, and that the peculiar appearance of adipose tissue in the embryo is due merely to the deposition of fat in embryonic connective-tissue cells. An intermediate position is taken up by Hammar (3), who has been able to distinguish in the embryo two distinct types of adipose tissue formation. In the one type the fat is deposited in ordinary connective-tissue cells which show no special arrangement in lobules; this he calls "secondary adipose tissue formation." The other type, which he calls "primary adipose tissue formation," proceeds in special groups of connective-tissue cells which show a distinct arrangement in lobules even before fat-globules become deposited in them. The cells, which are at first small, increase in volume owing to an increase in protoplasm and become polygonal. Fat is deposited in them in the form of small globules. We have, then, the embryonic gland-like fat tissue called by some authors the "primary fat organ." The fat-globules increase in size and eventually coalesce to form one large fat-globule, which gives the impression that this tissue is gradually being transformed into the ordinary type of adipose tissue. In the white rat, however, the process of "primary adipose tissue" formation remains in the embryonic stage, and we then get the "brown fat."

The glandular adipose tissue with which this paper deals is derived from the special groups of connective-tissue cells which exhibit the process called the "primary adipose tissue formation" by Hammar. In other words, it is histogenetically distinct from ordinary adipose tissue.

ON THE FUNCTIONAL SIGNIFICANCE OF GLANDULAR ADIPOSE TISSUE IN RELATION TO VITAMINES.

When rats or mice are fed on a diet of purified casein, starch, salt mixture, and olive oil, which is completely free from all vitamines, this glandular adipose tissue loses its fat and lipoid content, and in the terminal stages is completely free from either fat or lipoid. It has then the appearance of a glandular organ, as depicted in Fig. 5 (Plate II). The cells are large, rich in protoplasm, and filled with well-stained granular mitochondria. They contain on material which stains with either sudan or osmic acid vapour or osmic acid before or after bichromate fixation. Nor do they contain cell inclusions which are anisotropic or which are soluble in the ordinary fat-solvents, since paraffin sections of formol or Zenker-fixed material do not show any vacuoles in the cells. Frequently this tissue, especially the scapular tissue, is so deeply congested that it is purple in appearance and almost looks like a blood-clot. Sections of such a specimen reveal the very vascular nature of this tissue, for every cell can then be seen to be surrounded by capillaries. The tissue has the appearance of an internally secreting gland and resembles in fact the zona glomerulosa of the adrenal cortex. This resemblance is not an accidental or superficial one. For it is a noteworthy fact that the lipoids disappear also from the adrenal cortex as the result of withholding vitamines from the diet. There is, however, this difference—that the cortex is never completely freed from its lipoid content. A little always remains even when the surrounding peri-adrenal tissue has lost all its lipoids. The significance of this resemblance between the glandular adipose tissue and the adrenal cortex will be discussed presently. At present we wish to emphasise the following points. When, as the result of withholding vitamines from the diet, the animals become emaciated, the ordinary adipose tissue loses its fat deposit and disappears *qua* tissue, the cells reverting to the connective-tissue type of cell. But the glandular adipose tissue persists *qua* tissue and retains its glandular character which now in fact becomes unmistakable. Its morphological appearance, its highly vascular character, the independence of its lipoid content from the diet as compared with the ordinary fat metabolism and its functional relationship to the thyroid and adrenal glands, which will be discussed in great detail below, make it difficult to avoid the conclusion that this organ is an internally secreting gland. In any case since this tissue has been discovered and forgotten again and again, it seems desirable to emphasise its importance and its essentially glandular character by calling it "the lipoid gland" or "cholesterin gland."

Since this lipoid gland tissue is widely distributed throughout the body, some of it being in close connection with the large blood-vessels, its function cannot be determined by extirpation. But since the death of an animal on a vitamine-free diet coincides with an exhaustion of the contents of the gland, the conclusion seems obvious that the gland is itself rich in at least one of the vitamines and supplies the animal with it when the external supply is cut off. Experiments, which are at present in progress to test this point, appear to confirm this conclusion with reference to the fat-soluble accessory food-substance.

These observations raise a question of general importance in connection with the ætiology of the deficiency diseases. For they suggest that disturbances of the functional activity of this gland may constitute an important factor in eliciting the disease.

ON THE FUNCTIONAL SIGNIFICANCE OF THE GLANDULAR ADIPOSE TISSUE IN RELATION TO THE ADRENAL GLAND.

The adrenal gland lies embedded in the glandular adipose tissue, which is sometimes very firmly adherent to the adrenal gland itself. If a section of adrenal cortex with the surrounding fat is examined in polarised light, the double refracting material outside and inside the adrenal cortex has the same appearance. In fact in many cases a continuous band of double refracting material stretches across the field, and only on turning the analysing prism is it found that this band lies partly inside and partly outside the adrenal. Even if one did not know that this material contained cholesterin compounds both inside and outside the adrenal cortex, one would have no hesitation in concluding from the appearance of the picture in polarised light that the anisotropic material in the adrenal cortex is essentially the same as that in the periadrenal lipoid gland, and that a close functional relationship exists between the two. The adrenal cortex is known to contain a phosphatide, free cholesterin and cholesterin esters in addition to ordinary fat and fatty acids, and the same groups of lipoids may therefore be presumed to be present also in the glandular adipose tissue. The exact chemical nature of the phosphatide present in the adrenal cortex is not known.

The occurrence of cholesterin compounds in normal tissues is curiously restricted. Hitherto the adrenal cortex has been the only organ in which cholesterin compounds have been found to be present normally and constantly in considerable amounts. The corpus luteum is also stated to contain cholesterin compounds in certain stages of its development. To these two tissues may now be added the glandular adipose tissue. Both the adrenal and the ovary lie embedded in this tissue. While, therefore, the power to form or to serve as a store of cholesterin compounds is restricted to a very small group of cells in the organism, these few groups of cells happen to have a very close anatomical relationship. One cannot escape the suggestion that the glandular adipose tissue and the adrenal cortex are functionally related.

In this connection reference may be made in passing to the pathological conditions in which accumulations of cholesterin compounds occur, namely in the aorta in the experimental atheroma produced by feeding with cholesterin and in the large white kidney. Again, it is difficult to believe that it is a mere accident that both these organs have an intimate anatomical relationship with the glandular adipose tissue which normally stores and perhaps forms these substances.

This conception is not intended to imply that cholesterin fat tissue is equivalent to the adrenal cortex in every respect, but to suggest that they have a common or similar function in reference to the metabolism of cholesterin and the other lipoids present in the cortex, and to emphasise the necessity of taking into consideration the condition of this lipoid gland in these conditions in which changes in the lipoid content of the adrenal gland are observed. This conclusion is confirmed by the facts, already referred to above, that both the lipoid gland and the adrenal cortex retain their load of lipoid under those conditions under which the ordinary adipose tissue disappears, and that they both lose it when vitamines are withheld from the diet.

The recognition of the importance of the glandular type of adipose tissue throws fresh light on the metabolism of cholesterin, and more especially on the changes which occur in different conditions in the lipoids of the adrenal cortex. Although the exact function and destination of the cortical lipoids remain obscure as yet, certain definite conclusions can now be drawn with reference to their origin and to the significance of their changes in the cortex.

The work of Landau (10) (11), McNee (12), Hueck (15), Krylow (14) and others has shown that the lipoids do not represent the internal secretion of the cortex, as was at one time supposed. They are not formed in the cortex, but are taken up by the cortex from the outside. The adrenal cortex is therefore not the central organ regulating the metabolism of these lipoids and of cholesterin in particular, but rather the channel through which these substances pass to their destination, if not actually their destination. It remains to be seen whether the glandular adipose tissue represents the organ regulating the metabolism of these lipoids.

That the direction in which these substances travel in the cortex is from the periphery of the cortex towards the medulla has already been stated by us in a previous publication (16). The source of these lipoids is very clearly indicated in the condition of avitaminosis described in this paper. The adrenal cortex may be found still carrying its full load of lipoid while the surrounding periadrenal lipoid gland is already being depleted extensively. And when in the terminal stage the lipoid gland has completely lost its load of lipoid the adrenal cortex still retains a small amount.

The tenacity with which the adrenal cortex clings to the last remains of these lipoid substances illustrates another important point, namely that the absence of lipoids from the cortex is not compatible with life. Having examined the adrenals of hundreds of mice and rats, which had been subjected to a great variety of different conditions, I cannot recollect ever having seen a cortex extensively depleted of its lipoids except in animals which had died or were dying.

To avoid misunderstandings it may be as well to state that this does not mean that the lipoids always show an extensive depletion when an animal dies. And further it is necessary to draw a distinction between a mere diminution of the lipoid-load which may be found in a great many conditions, and the almost complete disappearance of these substances from the cortex, which is much less frequent.

In our previous observations on the functional activity of the adrenal gland in different conditions (16, 17), we have found that the disappearance of lipoids from the cortex is always accompanied by a paralysis of the gland. This is particularly striking in the condition of avitaminosis, where a subnormal temperature occurs some days before the terminal stages when the animal is obviously ill. This failure to maintain the normal body temperature is not due to an exhaustion of the medulla, which, although partly depleted, still shows a load of adrenin, but to an actual paralysis of the gland. The gland no longer responds to external cold by an increased secretion of adrenin, nor is it possible to produce a pyrexia when β-tetrahydronaphthylamine is injected. This association of disappearance of cortical lipoid and paralysis of the gland with resultant subnormal temperature has also been observed in some (not all) acute bacterial infections, such as gas gangrene (17).

The view is widely held at present that in all bacterial infections there is an extensive disappearance of the cortical lipoid. This view is based on a statement by Elliott, who was one of the first to examine the behaviour of the cortical lipoid in human post-mortem material in different pathological conditions. He stated that it disappeared with great rapidity in all acute fevers. The extensive observations of Landau and of Weltmann (13) show that this statement is too sweeping and that conditions are not so simple. Observations on human material give very conflicting results, and show that in many acute bacterial infections a normal or almost normal load of lipoid may be found post mortem. Nor is it possible to establish a relation between the nature of the bacterial infection and the effect on the cortical lipoid. A clearer insight is gained by observations on experimentally induced infections (17). It is then found that the disappearance of the lipoid is only a terminal phenomenon, which is preceded sometimes by a flooding of the cortex with lipoids. It is not the result of the bacterial infection as such, but occurs when the bacterial toxins have exhausted the adrenal gland. Thus a streptococcal infection which produces a circumscribed abscess but does not kill the animal does not produce a disappearance of the cortical lipoids or an exhaustion of the medulla. But when, with the same strain of streptococci, a cellulitis is produced by means of the kataphylactic phenomenon, the animals die, and the adrenals show an exhausted medulla and a cortex almost completely depleted of lipoids.

Whether all bacterial toxins have the same effect on the cortex remains yet to be determined. In a few experiments with tetanus no such effect has been observed. But even assuming that most bacterial toxins show this effect under experimental conditions, it can now be readily understood why human material gives conflicting results. In human post-mortem material the effect will vary with the actual cause of death, whether it has resulted from the pure action of the bacterial toxins, or whether it has been an accidental result such as asphyxia in diphtheria, hæmorrhage, or a toxic action on the heart or nerve centres.

A discussion has arisen as to whether the cortex or the medulla constitutes that part of the adrenal gland which is essential for life, and whether the symptoms following removal—or disease—of the adrenal are due to loss of cortex or to loss of medulla. Our histological observations on the adrenal gland (16) in different conditions of functional activity have shown that activity of the medulla is always accompanied by changes in the cortex, of which the changes in the lipoid are but one aspect, and that the two parts of the gland "act together, and are not, as has been suggested, two organs functionally quite independent of each other." We have only recently become aware that similar conclusions have been arrived at by Landau (11) and by Hueck.(15) Thus the latter writes: "For the normal functioning of the adrenal, medulla and cortex are equally important, and disease of one part acts not only through the diminution of its specific functions, but primarily through disturbance of the synergism."

SUMMARY.

In all types of mammals which have been investigated there exists a glandular type of adipose tissue histogenetically distinct from the ordinary adipose tissue. It has been described under various names, *e.g.* "primitive fat organ," "fat gland," "hibernating gland," "brown fat," "interscapular gland." In all species it has in the embryo a characteristic gland-like structure. In some species (white rat, tame mouse, hibernating animals) it retains this characteristic histological structure. But in most species the tissue acquires the appearance of ordinary adipose tissue soon after birth, so that the process has been looked upon as one of the stages leading to the formation of the adipose tissue of the adult organism.

It is shown that this tissue, which is very vascular, is functionally distinct from ordinary adipose tissue. The fatty material which it contains is rich in cholesterin compounds and other lipoids in addition to ordinary true fat. This load of lipoid is retained under conditions which bring about the disappearance of the ordinary adipose tissue. Evidence is given to show that it is functionally related to the thyroid and adrenal glands. A particularly close relationship exists between the lipoids of this tissue and those of the adrenal cortex. The significance of the changes of the lipoids in the adrenal cortex is discussed. The inter-dependence of the cortex and medulla of the adrenal gland for the normal functioning is again emphasised.

If vitamines are completely withheld from the diet the lipoids disappear from the adrenal cortex and from this tissue, which persists, however, as a tissue and exhibits the appearance of a very vascular endocrine organ. The existence of this tissue throws fresh light on the problem of deficiency diseases, since disturbance of the functional activity of this gland-like tissue will have to be considered as a factor in the ætiology of these diseases.

In view of its appearance, its function and its importance it is proposed to call this glandular type of adipose tissue the "lipoid gland" or "cholesterin gland."

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DESCRIPTION OF FIGURES.

Fig. 1.—Scapular lipoid gland of rat. Schridde preparation unstained. × 25 diam. The plate illustrates the lobular arrangement of the tissue as well as the differences in the size of the globules in which the lipoid material may be arranged within the cells. The lobules in the upper part of the plate consist almost entirely of the multiglobular variety; the lower part shows numerous uniglobular cells mixed with multiglobular cells. (Microphotograph.)

Fig. 2.—Human perirenal lipoid gland. From a child. × 240 diam. The left half of the plate is from a Zenker preparation, stained with hæmatoxylin and van Gieson. The right half from a Schridde preparation, unstained, of the same material. (From a drawing.)

Fig. 3.—Thoracic lipoid gland. Rat. Zenker preparation. Hæmatoxylin and van Gieson. × 250 diam. *Ao.* = aorta; *Adv.* = adventitia; *L. G.* = lipoid gland. (From a drawing.)

Fig. 4.—Perirenal lipoid gland. Mouse. Kolster preparation, stained for Altman's granules with aniline acid fuchsin and picric acid. × 1400 diam. The figure shows the relation of the mitochondria in the cells of the tissue to the lipoid globules. The latter have been dissolved out and appear in the figure as empty spaces. (From a drawing.)

Fig. 5.—Scapular lipoid gland. From a rat, dying as the result of being kept twenty-five days on a diet completely free from vitamines. Fixed in osmic vapour. Stained with Heidenhain's iron-alum hæmatoxylin after a previous treatment with 10 per cent. hydrogen peroxide in 80 per cent. alcohol. × 1200 diam. The tissue, which is now entirely free from fats or lipoids, is composed of large cells filled with granular protoplasm (mitochondria) and is very vascular and deeply congested. To the naked eye the tissue appeared purple when the rat was killed. (From a drawing.)

Figs. 2 to 5 were drawn with a camera lucida in the laboratory by W. Pilgrim.

THE MYCELIAL AND OTHER MICRO-ORGANISMS ASSOCIATED WITH HUMAN ACTINOMYCOSIS.

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Seventeen cases of actinomycosis came under treatment in the Inoculation Department, St. Mary's Hospital, during the years 1911–1914, and several more have been seen by the author since that time. Some observations made in connection with the earlier cases were being prepared for publication in 1914, but had to be laid aside in favour of military duties. This report has now been brought up to date and divided into two parts, of which one, containing the laboratory findings, is here presented.

Only those cases have been regarded as actinomycosis which showed suppurative lesions, the pus of which contained "granules" visible to the naked eye, composed of a feltwork of filamentous micro-organisms.

In restricting the use of the term to these cases, I have practically adopted the standpoint of Homer Wright(1), who first showed that such a limitation had the practical merit of isolating a group of infections which are attributable—in the great majority of cases, at any rate—to one and the same species of micro-organisms. In this way we avoid much of the confusion which has hitherto surrounded the subject of mycelial infections.

It is recognised that this arbitrary limitation is probably not entirely defensible, inasmuch as it would include certain distinct conditions such as "Madura foot" and other less common mycoses. When knowledge of the mycelial organisms is further advanced a more exact classification may be possible.

During the period covered by my observations of this disease, it has happened several times that mycelial organisms, showing no such aggregation into naked-eye granules, have been found in the discharge from various suppurative lesions, *e.g.* from an old empyema cavity, an acute suppuration in the region of the parotid gland, and an abscess of the breast. These organisms, when cultivated, were found to have characters quite different from those of *Actinomyces bovis* as described below. Cases of this kind have been excluded from the present series.

The presence of "clubs" ("ray-formation") at the periphery of the granules was also included by Homer Wright as a *sine qua non* in making the diagnosis of actinomycosis. In this I have not strictly followed him, for the reason that "clubs" were not looked for in several of the earlier cases of my series. In all the later cases (*i. e.* in at least 75 per cent. of the whole) these structures have been recorded as present. It would seem unlikely, however, that "club"-formation will prove a valuable distinguishing characteristic, since they have been shown, in recent years, to occur in at least two conditions quite distinct from actinomycosis, viz. in actinobacillosis, a purely bacillary infection of cattle (Lignières and Spitz(2)), and after the injection of killed tubercle bacilli into animals (Eastwood(3)).

NATURE OF THE CASES AND SOURCE OF CULTURES.

The 28 cases of actinomycosis which have come under my notice comprise 11 cases of the cervico-facial type, in whom the infection was first manifested on the lateral aspect of the jaw, and afterwards extended upwards or downwards, or in both directions; 7 thoracic cases, in whom the affection apparently originated in the lung-tissue or pleura; 8 abdominal cases, the majority of them having the original lesion in the appendix, or, at any rate, in the region of the cæcum; 1 case in whom both abdomen and thorax were involved (this case was unique in that the disease first manifested itself in two separate lesions, so far apart as the shoulder and the iliac fossa; the focus in the iliac fossa was reported to be extra-peritoneal, and there was no indication of an intra-thoracic origin for that on the shoulder); and 1 case in whom the back of the hand was the seat of the infection.

The course of the disease in nearly all the cases was very slow, extending over several months or even years. Of the 11 cervico-facial cases only 1 died (of cerebral abscess), and 9 are known to have recovered; of the 7 thoracic cases 5 are known to have died of the disease, and 2 have not been traced; of the 8 abdominal cases 6 have died, 1 recovered, and 1 is at present very ill; the case in whom both thorax and abdomen were involved died after three years' illness; the hand case made a rapid recovery.

Some notes on the treatment of this series of cases are being prepared for publication. Several of the cases have already been described by Mr. V. Z. Cope(4), to whom I am indebted for sending them to me, and for help in connection with their treatment.

In addition to the material derived from these cases, I have obtained cultures from the pus of three cases, kindly sent to me by Dr. Mervyn Gordon.

Both for diagnosis by films, and for cultural purposes, it has proved necessary to isolate the actual granules from the pus; separate mycelial filaments were very seldom found in film preparations of pus taken at random, and still more seldom was a growth of the organism obtained from such random samples.

When the granules, usually yellowish-white, about the size of a small pin's head, were not easily detected in the discharges on the surface of the wound, nor in the sample sent to the laboratory for examination, a small quantity of pus was vigorously shaken in a test-tube of water. All the ordinary elements of the pus were emulsified by this means, but the granules remained intact and were readily seen as they sank to the bottom of the tube, from which they were collected in a pipette.

To verify its mycelial structure and detect the "club-" or "ray-" formation, a granule was transferred to a microscopic slide, lightly crushed under a cover-glass, and examined, unstained, with a $\frac{1}{6}$th or $\frac{1}{8}$th objective. For more detailed examination another granule was crushed more thoroughly between two slides, stained by Gram's method and counterstained with weak carbol fuchsin.

To obtain cultures of the mycelium several granules were obtained as above, thoroughly washed in one or more changes of sterile water or broth in order to free the mycelium as far as possible from contaminating bacteria, and then crushed in a drop of fluid between sterile microscopic slides. Two glucose agar plates (P_H about 7·4) were inoculated from the mycelial suspension thus prepared, one being incubated aërobically, the other in a jar from which the oxygen was removed by the palladium chloride method (McIntosh and Fildes (5)). Before this anaërobic method was introduced, anaërobiosis was obtained usually by exhaustion of air, or by exhaustion plus absorption of oxygen with pyrogallic acid and soda. The results, however, when these latter methods were employed, were unsatisfactory in that growth often failed to occur, especially primary growths. I therefore relied more upon suspension cultures. These were made either (*a*) by aspirating the melted agar, after inoculation with an emulsion of a crushed granule, into a long piece of glass tubing, previously drawn out into a capillary at each end to permit of sealing with the inclusion of a negligible quantity of air (method of Vignal), or (*b*) more simply, by pouring the inoculated agar into a narrow test-tube and covering the top of the column with a layer of liquid paraffin or vaseline.

The mycelial colonies were easily recognised in such suspension cultures, and could be isolated by cutting across the glass tube and agar column.

The method recently described by Dr. Mervyn Gordon (6) of planting a granule into a tube of blood-broth under oil promises to secure primary cultures with greater ease than either of the above procedures. It proved successful in my hands with three of four samples of pus recently sent to me by Dr. Gordon, and in one instance a growth was obtained by this means when the surface culture failed to grow the mycelium. The method has one disadvantage, viz. that if pure cultures of the mycelium are required, these may be somewhat difficult to obtain from a primary mixed culture, in which the contaminating organisms frequently present (*e.g.* staphylococci, streptococci, and coliform bacilli) grow so much more quickly and profusely than the mycelium. For this subsequent isolation of the mycelium anaërobic plate-cultures are to be recommended, *the mass of mycelial growth being first washed in several changes of sterile water* in order to free it, as far as possible, from contaminating organisms.

THE MYCELIAL ORGANISMS.

The mycelial organisms isolated will first be dealt with, and afterwards certain bacteria found in association with these.

Growths of these organisms have been obtained from the granules of 27 cases and have ultimately been freed from associated bacteria. Three strains must be disregarded owing to insufficient records of their characters having been kept. Of the 24 remaining, 21 strains showed a general resemblance to each other, and may therefore be considered as one group (A). Two strains resembled the members of this group in respect of their morphology and preference for anaërobiosis, but differed somewhat in the character of their growths (Group B). One strain was frankly different from the common type (Group C). So far as memory serves to recall, the three strains which have been left out of account all conformed to the common type.

Group A.

The 21 strains of this group were filamentous organisms, the filaments being slender (0·5 to 1·0 μ), showing true branching, and seldom running straight for more than 20 to 30 microns. They retained Gram's stain, but often somewhat irregularly, so that deeply stained portions of the threads alternated with lightly stained portions (*vide* Fig. 1); at other times the deep staining was confined to mere dots within the thread. Recently Salomonsen's wet staining method has been applied to several strains with very satisfactory results, using fuchsin with carbolic acid and glycerine. A striking feature brought out in these preparations was the presence of very deeply stained dots at irregular intervals, sometimes 4 or 5 μ apart, at other points quite close together. Terminal thickenings and others of spindle shape have sometimes been seen along the threads, but neither spores nor the septate spore-bearing hyphæ recently described by Dreschler(7), in connection with the actinomyces of soil, have so far been demonstrated. No acid-fast strain has been met with. Growth of the organisms was always slow, being manifested only after three to eight days in primary cultures and two to six days in subcultures. It was never obtained at 22° C.

A strong preference for anaërobic growth was shown by all strains, but several were found to have become capable of some degree of aërobic growth after a period of frequent subculturing in broth under a column of liquid paraffin. Occasionally even primary aërobic cultures yielded one or two colonies after ten to fourteen days. Such aërobic surface growths always tended to grow down into the medium. With suspension cultures in glucose

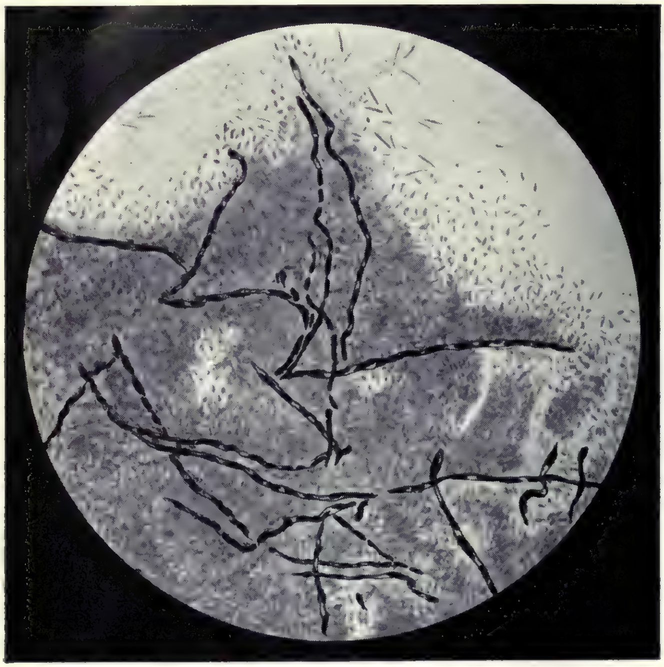

FIG. 1.—Crushed actinomycotic granule, showing mycelium of actinomyces surrounded by closely packed masses of *B. actinomycetum comitans.*

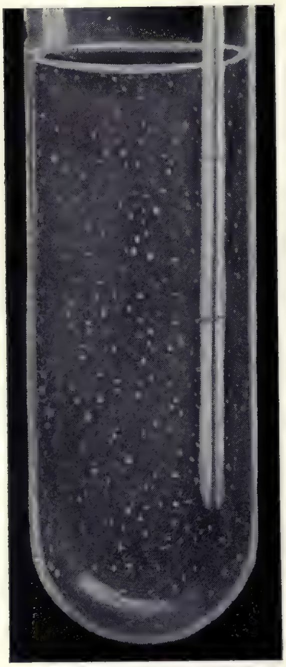

FIG. 3.—Broth culture of *B. actinomycetum comitans,* showing pin-point colonies adherent to the glass.

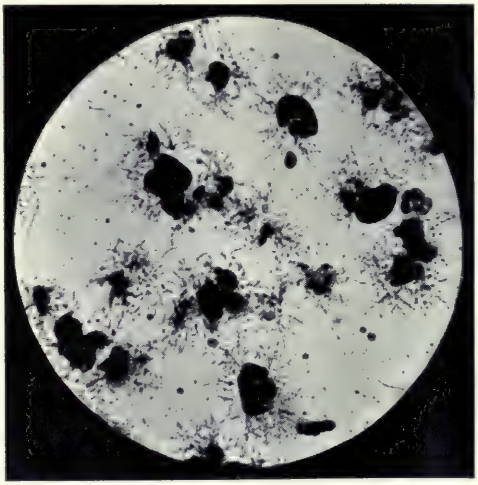

FIG. 2.—Surface colonies of *Actinomyces bovis* on glucose agar, 6 days old. × 55.

agar, it was noticed on several occasions that a dense band of small colonies would develop about half an inch below the top of the column, while in the deeper parts of the column the growth was much more scattered.

Cultures of all strains were obtained on ordinary nutrient agar or broth, but were much more luxuriant if ½ or 1 per cent. of glucose had been added to the medium.

A series of tests with nutrient broths of different hydrogen-ion concentrations showed that when the P_H lay within the range 6·7 to 7·7 good growths were obtained, and that the optimum was from 7·3 to 7·6.

Surface cultures were usually obtained without difficulty when the palladium chloride method of securing anaërobiosis was employed, otherwise they were frequently unsuccessful. Young colonies, under low magnification, showed a margin of irregularly radiating processes (Fig. 2). When more fully grown they formed dull white, heaped-up masses, which were very adherent to the medium and were not easily broken up. The typical appearance of a fully-grown surface culture is well shown in a photograph published by Wolff and Israel(8) (Plate I, fig. 1).

In suspension cultures the colonies were opaque and quite irregular in shape, suggesting rather the appearance of a cauliflower in miniature. They were thus in sharp contrast to the regularly formed lenticular colonies of staphylococcus, with which they were often mixed in primary cultures.

In fluid media (usually glucose broth) growth occurred always at the bottom of the tube in the form of opaque, dull white masses, rather suggestive of bread-crumbs, which were not easily broken up. The broth remained clear, but would sometimes show a flocculent deposit when its reaction became acid after five to ten days.

In tubes of uncooked white of egg, or a mixture of yolk and white, little if any growth was obtained.

In human serum, of which the anti-tryptic power had been neutralised by the addition of trypsin, a fairly free growth occurred. Very little growth was obtained in unaltered fresh serum.

The viability of these strains was somewhat slight. They were always killed by exposure to 60° C. for an hour, and surface cultures appeared to be sensitive also to desiccation. Even when frequently subcultured they were apt to die out.

Cultures on egg medium, when kept moist, have remained alive for four to six weeks, and one strain lived for four weeks on the surface of glucose agar when kept in the ice-chest.

Glucose broth cultures did not usually survive longer than fourteen days at 37° C.

Identity of the strains of Group A with the Actinomyces bovis *of Wolff and Israel.*—Comparison of the characters described above with those attributed by Wolff and Israel(8) to the organisms which they isolated from two cases of actinomycosis, and also with the account given by Homer Wright of the 13 strains isolated by him in America, leaves little doubt that the 21 strains here in question showed a very close resemblance to the strains of these authors. Without entering upon vexed questions of nomenclature, I have therefore followed the lead of Wolff and Israel and applied to these 21 strains the name "*Actinomyces bovis*." This seems to have been introduced in 1877 by Harz, who first described an organism apparently identical with that of Wolff and Israel.

These authors noticed a tendency of their strains to revert to a chiefly bacillary habit of growth in artificial media. One or two of my strains have shown this tendency, but it has not been the rule. Wolff and Israel also seem to have found their strains somewhat more hardy than mine have proved; this, however, may be accounted for by the fact that these authors largely used egg medium, whereas I have employed chiefly media containing a considerable amount of sugar.

Group B.

Unfortunately my notes of these two strains, which were isolated just before the war, are not adequate for a satisfactory description. Both were recovered from clinically typical cases of actinomycosis, with granules in the pus. In one case the illness started as an acute attack of appendicitis.

Both strains resembled *Actinomyces bovis* in their preference for anaërobiosis, their slight viability, and in their morphological characters when first isolated. One of them differed chiefly from the strains of Group A in the character of its growth on the surface of glucose agar. The second strain grew badly on glucose agar, and in glucose broth produced a uniform turbidity with a sediment—not bread-crumb-like masses. On blood agar the mycelium grew fairly readily in small greyish colonies, which were slightly raised and did not stick to the medium. No aërobic culture was obtained.

The second and third generation in broth showed almost entirely bacillary forms.

Group C.

This strain (Taylor) was recovered from a granule found in pus from an old-standing thoracic sinus. The illness had commenced with empyema four years previously, and this had been followed by several abscesses in the chest-wall. When seen by the author the patient was discharging occasional granules from a lesion near the xiphisternum. His general health was very good.

A film preparation of a granule showed a mass of mycelium of which the elements ran rather straighter than those of *Actinomyces bovis*, but were, in other respects, similar to that organism. Unfortunately the granule was not examined for "ray"-formation.

Cultures yielded a single colony of an *aërobic* mycelial organism, this colony developing in the water of condensation. A few colonies of diphtheroid bacilli also grew. Subcultures of the mycelial colony on glucose agar gave several large flat rosettes (3 to 5 mm. in diameter), which grew deeply into the medium and were extremely tough. In gelatine at 37° C. they formed a large, spongy mass. The cultures died out during the writer's absence for three weeks, and so were not examined further.

THE GEOGRAPHICAL DISTRIBUTION OF *ACTINOMYCES BOVIS* INFECTIONS.

The predominance of *Actinomyces bovis* in the author's series of cases is of interest as bringing the experience of English cases into line with that of several other countries. As stated above, Homer Wright cultivated this organism from 13 consecutive cases of human actinomycosis in America. Of 29 Japanese cases from which cultures were obtained, 28 gave strains of this type (Shiota (9)); Schukewitsch (10) isolated 22 similar strains derived from 25 cases in Russia; and Harbitz (11) obtained 20 strains in Norway. Finally, Pinoy (12), working in Paris, described 13 strains out of 16 isolated from cattle, and 2 out of 3 from human actinomycosis, as agreeing with this type, to which he gives the somewhat cumbrous name "*Cohni-streptothrix Israeli.*"

THE SOURCE OF THE INFECTION IN HUMAN ACTINOMYCOSIS.

The established idea that the "ray fungus" is conveyed to man from vegetable sources, *e.g.* grasses, soil, etc., should be viewed with considerable scepticism.

This concept appears to have been based upon the view that the common cause of actinomycosis is an *aërobic* organism of the type described by Bostroem (13) and others, which is more or less akin to certain moulds recoverable from vegetable sources. The work of Homer Wright and others quoted above has shown, however, that the infective agent in most cases of actinomycosis is not of this type, but is the *anaërobic* organism here referred to as *Actinomyces bovis* (Group A of the author's series); and no one, so far as I am aware, has yet recovered an organism of this latter type from any vegetable or other "natural" source, *i. e.* outside the animal body. When we consider the slight viability of *Actinomyces bovis*, its apparent inability to form spores or to propagate the mycelial generations except at body temperature, there seems little reason to expect that it will be recovered from "natural" sources.

With a view to testing this hypothetical infection by the "fungus" when conveyed into the tissues on vegetable material, a few experimental inoculations of rabbits were carried out. Sterile oats were impregnated with a fluid culture of *Actinomyces bovis*, and were then introduced into the pleural and peritoneal cavities. Actinomycotic infection was not established in any instance.

Finally, before leaving this view of the question, it may be remarked that only 1 of the 28 cases of which I have had experience gave a history that at all suggested this traditional mode of infection. The patient was a boy under the care of Mr. Cope, who has reported it in his paper (Case 1). A piece of grass was discharged in this case from an abscess at the angle of the jaw.

Another, and seemingly more credible, view as to the origin of the disease has recently been gaining ground. It does not attempt to explain from what source or in what way *Actinomyces bovis* is originally conveyed to the human body, but suggests that it is a frequent inhabitant of the alimentary tract of man, and that in certain occasional circumstances, which are not yet clear, it is carried into the tissues and is enabled to establish itself there.

The evidence in favour of this view is as follows:

Lord (14) has called attention to the fact that in carious teeth, and also in tonsillar crypts of normal persons, one may frequently find filamentous organisms having the morphology and staining reactions of *Actinomyces bovis*; and further, he has succeeded, by inoculating such material intraperitoneally into animals, in reproducing granulomatous lesions which resembled those of actinomycosis in that they contained granules composed of a feltwork of the mycelium and surrounded by typical clubs.

The present writer also found Gram-positive filaments not unlike those of actinomyces in each of six carious teeth examined, but was not able to carry the investigation further at that time. It would seem very desirable to isolate these filamentous organisms from a series of teeth and determine whether they can be identified with *Actinomyces bovis* by serological tests.

In support of the possible dental origin of the disease in certain patients, one of the cases reported by Mr. Cope and included in the present author's series was of peculiar interest. The patient had injured his knuckle by knocking out an opponent's tooth three months before coming to hospital. An acute infection ensued, with lymphangitis; this subsided, and was followed, after a few days, by the development of a subacute actinomycotic lesion.

A very similar case has recently been reported in America (McWilliams (15)).

AGGLUTINABILITY AND AGGLUTINOGENIC PROPERTIES OF *ACTINOMYCES BOVIS*.

The author's observations in respect of these properties of actinomyces are very incomplete, but inasmuch as very little has been written on the subject hitherto, it is thought perhaps worth while to record the following points in summary form: (*a*) The serum of heavily-infected patients usually gives a coarse agglutination with suspensions of *Actinomyces bovis*. Such sera were often effective in a dilution of 1 in 500, sometimes in dilutions as high as 1 in 4000.

Most of the patients with relatively slight infections, *e.g.* the cervico-facial cases, failed to show such agglutination. The reaction will probably be of little use for diagnosis, since cases usually have open lesions in which the mycelium can be found by the time the serum has developed agglutinating power.

(*b*) Similar coarse agglutination is obtained with the sera of rabbits inoculated with a vaccine of *Actinomyces bovis.* Two doses of 500 and 1000 million mycelial fragments under the skin, followed by three doses of 500, 1000 and 2000 million fragments respectively into the blood-stream, at two or three days' interval, sufficed to obtain a serum agglutinating up to a dilution of 1 in 1000.

(*c*) Fine agglutination, occurring very slowly, is sometimes seen with normal sera, even when diluted 50- or 100-fold, but there is usually no difficulty in distinguishing between this and the coarse agglutination which occurs within one hour or two hours at 50° C. with the sera of infected persons or animals. Spontaneous agglutination of the suspension of actinomyces was also noticed at times, particularly if a 0·85 per cent. or 1 per cent. salt solution were employed. Suspensions in weaker salt solutions have been free from this defect.

(*d*) The most satisfactory suspensions of actinomyces for agglutination tests were prepared by thoroughly washing the growth obtained from a broth culture, drying it on filter-paper, and then rubbing up the flaky powder in a mortar with 0·4 per cent. sodium chloride solution.

Serological types of Actinomyces bovis.—The preparation of agglutinating sera to several strains of this organism, with a view to testing them against all available strains, had just been begun when the work was interrupted by the war. It has not yet been determined, therefore, whether there exist several different serological types of *Actinomyces bovis.*

Two such sera have been kept, and others are in course of preparation.

The sera of infected patients were tested from time to time against several strains, and showed a certain amount of cross-agglutination. The results were, however, sometimes suggestive of serological differences. One such observation is perhaps worth recording: The serum of a patient, D. R—, although it agglutinated the homologous strain, failed to agglutinate another strain, A. W—; while the serum of the patient A. W— agglutinated the strain D. R— as well as the homologous one. This serum also agglutinated four other strains.

ON CERTAIN BACTERIA COMMONLY FOUND IN CLOSE ASSOCIATION WITH ACTINOMYCES.

Actinomycotic granules are usually surrounded by an envelope of closely packed leucocytes. If this envelope is washed away by shaking the granules in water, and the core remaining is then crushed between two slides, stained by Gram's method and counter-stained with weak carbol-fuchsin, the felt-work of Gram-positive mycelium will often be seen upon a background which has taken the counter-stain, but in which no detailed structure can be made out; it appears as a pink or red blur.

This counter-stained background is in reality a densely compacted sheet of very minute Gram-negative cocco-bacilli, as may be ascertained by following it out to a point where the sheet has been lacerated in crushing the granule. At such a point it will frequently be possible to trace the fusion of the multitude of individual cocco-bacilli (*vide* Fig. 1).

My attention was first directed to these organisms in 1912 in finding that a multitude of tiny colonies, quite distinct from the mycelial colonies, had grown in an agar suspension inoculated from a crushed granule which had shown only mycelial elements on casual examination of a film. A smear of these tiny colonies showed them to be composed of Gram-negative cocco-bacilli. On going back to the film made from a granule and looking carefully at the thinner parts of it, great numbers of lightly stained Gram-negative elements could be made out and their fusion into dense sheets could be determined at some points.

Since that observation was made the presence or absence of these organisms in the granules has usually been noted. Out of thirty patients whose granules have been thus examined, they have been found in no less than twenty-four, and in more than ten instances the organisms have been cultivated as well as seen in films. Usually, but not invariably, they were present when the patient first came under treatment, and persisted throughout the course of the disease; in one case they were not found until the patient had been under observation for nearly a year, but from that time were constantly present. As a rule they occurred, as described above, in the form of dense masses, giving the appearance of a matrix about the mycelial feltwork of the granules; I have never detected them free in the pus.

Morphology.—When seen in films of crushed actinomycotic granules, and also in smears made from cultures, these minute organisms most commonly have the form of micrococci (diameter about 0·3 to 0·5 μ), or very short round-ended bacilli (0·8 to 1·5 μ in length). On this account they were at one time referred to in the laboratory as "*Micrococci pseudo-melitensis.*"

Longer bacillary forms are also frequently seen and may perhaps represent a distinct type, but no definite evidence of this has been obtained.

The formation of short chains has occasionally occurred in broth cultures, but seems to be exceptional. Capsules have not been demonstrated. The organism is non-motile.

Gram's stain is never retained, and if neutral red is employed as the counter-stain the organism may not be detected, for it takes that dye very badly. Carbol fuchsin is a better counter-stain, but even that is not taken up so readily as by most bacteria. With Leishman's modification of Romanowsky's stain a tendency to bi-polar colouring has sometimes been noted.

Cultural characters.—The outstanding characteristic of this organism is the tenacity with which the individuals cling to each other. This has already been seen in connection with cultures of the microbe in the body, *i. e.* in the actinomycotic granule, and it is no less manifest in cultures grown outside the body. The colonies produced on the surface of agar are not only very difficult to break up, but also to remove from the medium. And in fluid cultures growth is characterised by the formation of star-like colonies which adhere with extraordinary tenacity to the walls of the tube (Fig. 3), so that they are scarcely to be removed by mere shaking.

Growth occurs equally well under aërobic or anaërobic conditions, but is somewhat precarious. Primary cultures from crushed granules have very frequently failed to grow the microbe, possibly because it had been killed by the great concentration of leucocytes around the granule. The successful cultivations, when obtained, were made upon ordinary media, simple nutrient agar or glucose agar; one strain, however, required blood agar at first, and another was certainly favoured by the inclusion of serum in the medium.

No growth has occurred at 22° C.

Surface colonies become visible after twenty-four hours' incubation and usually increase in size for two or three days—seldom, however, exceeding 1 mm. in diameter. They are discrete, hemispherical, and glisten with the lightest suggestion of a blue tinge. As described above, they are dislodged with difficulty from the medium and are not easily broken up.

In broth cultures the colonies develop, as was explained above, on the sides of the tube, not exclusively at the bottom. They are usually about 0·5 mm. in diameter when full grown, but may be slightly larger. After several subcultures one strain was observed to give a uniform turbidity in broth.

Glucose, lactose, mannitol, dulcitol, saccharose and dextrin respectively, were fermented by one strain, the only one tested—acid being formed but no gas.

Viability.—This is apparently slight. Cultures are killed by exposure to 52° C. for half an hour, or for two days in the ice-chest. Surface cultures on agar have survived on the bench for two to three weeks, suspension cultures a somewhat shorter time.

Pathogenicity for man.—It is difficult to determine what part, if any, is played by this microbe, as distinct from actinomyces, when both are present in an actinomycotic lesion. The cocco-bacillus has not been found in any lesion independently of actinomyces.

When a living suspension of the organism, which had been frequently subcultured (and was therefore, perhaps, avirulent) was on one occasion introduced into the skin of a normal individual no effect was produced.

The investigation of serum reactions (opsonic and agglutination) in infected patients has not been of much value. Great difficulty is experienced in obtaining satisfactory suspensions for these purposes from the scanty and tenacious cultures. High opsonic values (1·4 to 1·8 approximately) have been recorded on several occasions.

Vaccines of the organism have sometimes induced mild febrile reactions in doses of over 40 millions.

Pathogenicity for laboratory animals.—Of three rabbits inoculated intravenously with a culture of this organism, the quantities varying from 300 to 15,000 millions, two died within twenty hours. The lungs, kidneys and spleens showed some congestion, and the leucocytes in the blood-stream were crowded with phagocyted organisms.

Two rabbits inoculated into the peritoneal cavity and subcutaneous tissue respectively, were killed, and no evidence found of either a local or general infection.

Of six guinea-pigs inoculated intraperitoneally, two died with diffuse peritonitis and some white nodes in the omentum, but no granule-formation was detected. Another died after thirty days with congestion of the tunica vaginalis but no peritonitis. Guinea-pigs inoculated subcutaneously developed an indurated inflammatory reaction which did not go on to abscess-formation.

References to this cocco-bacillus in the literature.—The paper by Wolff and Israel (8) (1891), which first described the cultural characters of *Actinomyces bovis*, contains some discussion as to the nature of certain pleomorphic bodies, "possibly micrococci," which had been observed by them as well as by other workers, mixed with the mycelium of actinomycotic granules. It seems unlikely, however, that these "micrococci" can have been identical with the bacteria described above, since the latter are always frankly Gram-negative, whereas Wolff and Israel's "micrococci" are described as Gram-positive. These "micrococci" do not appear to have been cultivated.

The only paper I have met with which describes the same organism as the one here in question is that of Klinger (16), who, working in Zurich, found it in the granules of four cases of actinomycosis, always along with actinomyces. He described its cultural characters and proposed for it the name of *B. actinomycetum comitans*, by which it will be referred to hereafter in this paper.

DISCUSSION AS TO THE IDENTITY OF *B. ACTINOMYCETUM COMITANS* AND AS TO THE SIGNIFICANCE OF ITS ASSOCIATION WITH ACTINOMYCES.

The bacterium has not, so far as I am aware, been identified with any other species. Nor has it been met with in any other habitat than the actinomycotic granule.

I suspected at first, on account of certain resemblances in its morphological and cultural characters, that it might prove to be identical with the actinobacillus of Lignières and Spitz. These authors described in 1902 a disease occurring among Argentine cattle which resembled actinomycosis in giving rise to suppurative lesions, the pus of which showed granules with well-marked "ray-formation." The disease, however, differed sharply from actinomycosis clinically by its predilection for attacking lymphatic glands, and, bacteriologically, by the absence of mycelium from the granules, which were composed exclusively of minute bacilli. The term "actino-bacillosis" was applied to the disease, and "actino-bacillus" to the causal organism. It has been shown by

F. Griffith (18) (1914) that the great majority of so-called actinomycosis lesions of cattle in this country are, in reality, due to actino-bacillosis, the bacillus recoverable from them showing a close resemblance to that of Lignières and Spitz. One human case of infection by this bacillus has been reported by Ravaut and Pinoy (17) in Paris. The suspected identity of *B. actinomycetum comitans* with this actino-bacillus has not, however, been established. On examining cultures of the actino-bacillus, kindly shown to me by Dr. F. Griffith, it was found that this microbe produces a much more abundant surface growth on agar, and in broth a uniform turbidity with a scum on the surface.

To the question, What is the significance of the frequent association of the bacterium with actinomyces in human actinomycosis? no answer can at present be given.

The following four hypotheses have suggested themselves and may be briefly discussed: *Hypothesis* 1.—B. actinomycetum comitans *is very widely distributed in nature, and therefore finds easy access as a secondary infection to the open lesions of actinomycosis.* This suggestion may be put aside for the following reasons: The bacterium is a quite unfamiliar species, and has not been met with, so far as I am aware, as a secondary invader of any other open wounds. Further, the microbe has been found, in at least one instance, in the pus of a primary actinomycotic abscess (unopened).

Hypothesis 2.—B. actinomycetum comitans *is widely distributed in nature, but establishes itself only in tissues which have been invaded by actinomyces, the previous growth of that fungus having created conditions peculiarly favourable to the bacterium.* In the expectation that a favourable influence of this kind, if strongly exerted by the growth of actinomyces in the body, might also be evidenced in some measure by its cultivation *in vitro*, the following experiment was performed:

A fully-grown suspension culture of *Actinomyces bovis* in glucose agar was melted down, its alkalinity restored to correspond with that of a control tube, and thereafter sloped. Upon this tube, and a control, *B. actinomycetum comitans* was now planted from a common source. A similar experiment was conducted with liquid medium. In both cases the growth obtained with the medium which had previously yielded a crop of actinomyces did not exceed, but rather fell short of that obtained with the fresh (control) medium.

We have, then, no evidence in favour of this hypothesis, which appears unlikely to offer the solution of the problem.

Hypothesis 3.—B. actinomycetum comitans *occurs along with actinomyces in "nature,"*—i.e. *before the latter gains access to the human body—and the infection is consequently a double one.* The association of the two organisms in nature is regarded, on this hypothesis, as independent of any genetic connection between them. This hypothesis is scarcely yet ripe for discussion, since we do not yet know what is the source of actinomycosis in nature, nor how its transference to man comes about.

Hypothesis 4.—*The association of the two organisms in the human lesions is conceived to depend upon some genetic connection between them.* Such a suggestion is not entirely new. Dunbar (19) has described microscopical and cultural observations which suggest the formation of bacillary organisms from certain algæ. He held the view that cholera-like vibrios, plague bacilli and other organisms actually developed from moulds and algæ.

The question whether a similar concept can be applied to the case of actinomycosis must necessarily be held over for future discussion. The research which such a discussion must draw upon is beset by the especial difficulties which attend all experiments pointing to the transformation of microbic species. And particularly in the case now at issue, where both the bacillus and its colony are so small as to easily escape recognition, most scrupulous care will be necessary in order to disarm the possible criticism that the bacillus has been present in, and carried over from, the cultures of actinomyces employed.

For the present only prefatory observations may be recorded:

(1) The bacilli have been found present in abundance in the granules of an unopened primary abscess. Only this one opportunity has yet occurred to examine material from a primary abscess.

(2) 80 per cent. of cases specifically examined for it have shown the bacilli to be present, but in some of these it has only been found in the discharges at irregular intervals. One case constantly failed to show the bacilli over a period of twelve months.

(3) By direct examination of young growing plate cultures of *Actinomyces bovis* under high magnification, Captain Douglas has occasionally observed the inclusion within the mycelial filaments of small bodies comparable in appearance to the *B. actinomycetum comitans*, and at once suggestive of analogous findings described by Dunbar.

(4) By an indirect method, the investigation of specific antibodies, evidence of the kinship of the two organisms has also been sought, but has not been found. The opsonic power of the serum of an actinomycosis patient was tested to both the ray fungus and *B. actinomycetum comitans*. The serum was then incubated with a thick emulsion of the fungus, which was thereafter removed by centrifugalisation. Upon re-testing the serum with the phagocytic emulsions of the organisms, it appeared that while the opsonic power for actinomyces had been almost entirely lost, that for *B. actinomycetum comitans* remained unchanged.

THE SECONDARY INFECTIONS OF ACTINOMYCOSIS (APART FROM INFECTION BY *B. ACTINOMYCETUM COMITANS*).

An open actinomycotic lesion is very rarely infected only by actinomyces. In the cervico-facial cases staphylococcus will usually be found and sometimes also streptococcus, but these infections have not appeared to play an important part in the progress of the disease. In the abdominal cases, on the other hand, secondary infection from the intestinal tract frequently occurs, giving to the discharges the extremely offensive smell characteristic of proteolytic digestion by bacteria. Anaërobic bacteria, diphtheroid and coliform bacilli are frequently found in this condition, and since their presence generally implies deep suppurative foci which it is impracticable to drain effectively, a grave degree of toxæmia is apt to occur and a fatal issue is the rule.

SUMMARY.

(1) Cultures of mycelial organisms were obtained from the granules of twenty-four cases of human actinomycosis. Twenty-one of the twenty-four strains conformed in their general characters to the anaërobic type described by Wolff and Israel (*Actinomyces bovis*), and later, in greater detail, by Homer Wright; two strains were also anaërobic organisms but with slightly different cultural characters; and one was a quite different, aërobic type.

(2) All strains of *Actinomyces bovis* which were tested showed coarse agglutination with the serum of heavily infected patients. The serum of rabbits inoculated with the organism also gave a strong agglutination reaction.

(3) The current hypothesis that actinomycosis is directly conveyed to man from vegetable sources or from soil is held to be probably false. Certain evidence is put forward which suggests that the organism is a common inhabitant of the human alimentary tract.

(4) Attention is called to the fact that, in addition to the mycelial feltwork always found in actinomycotic granules, there are frequently immense numbers of minute Gram-negative cocco-bacilli. In 80 per cent. of the cases these organisms have been found at some period of the disease; and in about a dozen instances they have been cultivated on artificial media. They are of unfamiliar type, and have not, so far as is known, been met with in any other circumstances. It was proposed by Klinger, who reported the cultivation of similar organisms from four cases of actinomycosis in 1912, to apply to them the name *B. actinomycetum comitans*. This name is here employed.

The association of *B. actinomycetum comitans* with actinomyces in the granules is discussed but no satisfactory explanation has been arrived at. The findings are somewhat suggestive of a genetic relationship between the bacillary organism and actinomyces, analogous to that which Dunbar claimed to have demonstrated in the case of cholera-like vibrios and algæ.

In conclusion I wish to acknowledge the valuable help given to me by Sir Almroth Wright and Capt. Douglas, under whose guidance many of the cases were treated and the laboratory work carried out.

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VARIATION IN AGGLUTINABILITY OF BACTERIA ASSOCIATED WITH VARIATION OF CULTURAL CHARACTERS.

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MUTATION of organisms belonging to the group of Gram-negative intestinal bacilli has been studied by various observers, but mainly with regard to variation of biochemical reactions. These variations have been observed occurring spontaneously and also under the influence of inhibitory agents in culture medium.

Massini (1) originally described a non-lactose-fermenting Gram-negative bacillus (*B. coli mutabilis*) which on Endo-agar developed lactose-fermenting mutants, represented by red papillæ on the pale colonies of the original strain, and, after further subculture, as red colonies. Penfold (2, 3) also described mutation of *B. coli* types growing on monochloracetic-acid-agar, the variant being characterised by absence of gas production in certain fermentation tests. Such variants, however, show no difference from the original strains as regards their serological characters.

Recently Fletcher (4) has described atypical forms of *B. paratyphosus B* and *B. aertrycke* occurring under natural conditions, which in artificial culture exhibited variation both in cultural and serological characters and reverted to the typical form. The original strains were capsulated bacilli, produced slimy or "mucoid" colonies, and were much less agglutinable than the classical strains; after subculture, however, typical bacilli developed from the mucoid strain, and these reacted to full titre with a specific agglutinating serum. Fletcher has also described the same variation in the case of strains of dysentry bacilli.

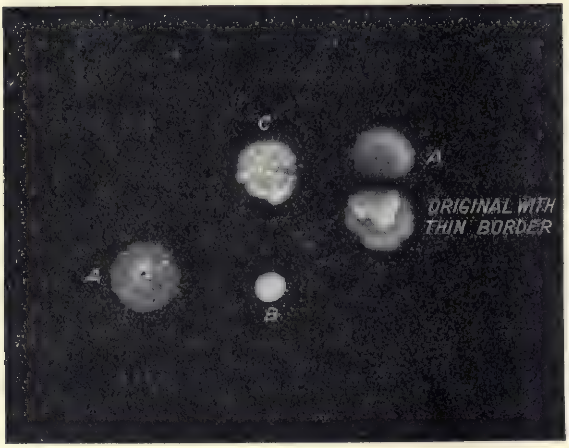
In the course of certain extended observations on the *B. coli* group a similar variation has been noted. It was found that a *B. vesiculosus* (MacConkey) strain which produced on agar plates large, raised, thick, opaque, slimy or "viscous" colonies, and grew in the form of a thick, white, glistening, viscous growth on agar-slope cultures, when grown on agar containing brilliant green in such amounts as to produce slight inhibition of growth, developed variants from the original type as regards colony characters; it was also noted that these variations were associated with differences in agglutinability by a serum specific to the original strain.

The general characters of the original strain were as follows: Gram-negative, non-motile bacillus; not liquefying gelatine; fermenting, with gas production, glucose and lactose, but not dulcite, saccharose, adonite, inulin, or inosite; producing indol from peptone; not yielding a Voges and Proskauer reaction; *i. e.* corresponding to *B. vesiculosus* (MacConkey). The colony characters have already been referred to. When plated out on nutrient agar containing 0·06 c.c. of a 1 : 10,000 watery solution of brilliant green to 5 c.c. of agar, it developed two different types of colony—(*a*) thick, viscous colonies corresponding to those of the original strain, and (*b*) thin, transparent, non-viscous colonies.

Subcultures from the thin colonies reproduced on ordinary culture medium similar thin, non-viscous colonies; thus from the original growth a variant strain could be isolated differing in cultural characters from the original strain and remaining true to type even after repeated subculture. In all the other general characters the new strain corresponded to the original.

The original culture did not show variation in colony characters when plated on ordinary medium. It had been used as a type in certain serological investigations, and there could be no doubt as to its purity since the strain had been originally obtained after three successive platings and subculture from single colonies. An agglutinating serum had been prepared by immunisation of a rabbit.

Subcultures from a thick viscous colony on the brilliant green agar plate replated on the same medium again yielded different types of colony, viz.: Type A—thin, non-viscous colonies as in the first experiment; Type B—small, thick, opaque, markedly viscous colonies; Type C—large, thick, opaque, non-viscous colonies; and also large opaque viscous colonies corresponding to those of the original strain. The different appearances of these colonies are shown in the photograph.



Colonies on agar plate after 4 days at 37° C.

Primary subcultures from colonies A, B and C reproduced the respective colony characters; repeated subcultures remained true to type and there was no tendency to reversion on subculture. On microscopic examination types B and C were more coccoid in shape, while A represented typical bacillary forms. By overstaining with Leishman's stain (ten minutes with the pure stain and thirty minutes with a 1 : 2 dilution in distilled water) a thick, somewhat irregular capsule was demonstrated in the case of B, while A and C were apparently non-capsulate. Hiss's method failed to show a distinct capsule. It was found that after three or four days' incubation, from some of the large, thick, viscous colonies representing the original strain, thin transparent borders were developed and appeared to correspond to the thin colony variant; subcultures from these borders reproduced thin colonies of type A, which remained true to type. It was also found later that subcultures from the large, thick, viscous colonies on the original brilliant green agar plate, replated on ordinary medium, likewise developed colonies from which the type A variant grew out in the form of a thin border. Thus from the original strain three different variants had been developed, as regards growth characters on solid culture medium; the biochemical reactions and other cultural characters of the original strain were reproduced in the new types, and it was shown how the variant A actually developed from the original type of colony.

Agglutination tests were carried out with the antiserum to the original strain, and striking differences in agglutinability were noted between the different types. The end-titre of the reaction with the original strain was 1 : 1600; type A was agglutinated by the same serum in dilutions up to 1 : 10,000; C displayed a lesser agglutinability than the original strain, the end-titre being 1 : 1000; in the case of B the end-titre was 1 : 3000 (Table I).

These results are of special interest as demonstrating variation in agglutinability associated with variation of other characters. On retesting these variants after several subcultures the agglutination results were found to be constant.

Similar observations were made with another *B. vesiculosus* strain to which an agglutinating serum had been prepared. The original strain grew in the form of large, white, opaque, non-viscous colonies. On brilliant green agar two types of colony were observed: (1) Those which represented apparently the original strain, and (2) thin transparent colonies resembling variant A of the first experiment; subcultures from these remained true to type as regards the new character, but did not differ in other general characters from the original strain. Agglutination tests yielded results which corresponded to those found in the first experiment; thus the thin colony variant again proved much more agglutinable than the original strain (Table II). Similar mutations as regards colony characters were observed with two other *B. coli* strains, and the variants remained true to type even after repeated subculture. It is to be noted that many *B. coli* strains grown on medium containing a slightly inhibitory concentration of brilliant green may develop thin colonies, but on subculture they revert to the original type.

TABLE I.

Agglutinating Serum to B. vesiculosus *Strain H.*

	Serum dilutions.										
	1 : 400.	1 : 800.	1 : 1000.	1 : 1200.	1 : 1600.	1 : 2000.	1 : 3000.	1 : 4000.	1 : 6000.	1 : 10,000.	1 : 20,000.
Original strain	++++	+++	+++	+++	++	—	—	—	—	—	—
Variant A . .	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	—
„ C . .	+++	+++	+	—	—	—	—	—	—	—	—
„ B . .	++++	++++	++++	++++	+++	++	+	—	—	—	—

Reading after 4 hours at 37° C. Emulsions all of equal density.

TABLE II.

Agglutinating Serum to B. vesiculosus *Strain L.*

	Serum dilutions.										
	1 : 800.	1 : 1000.	1 : 2000.	1 : 3000.	1 : 4000.	1 : 6000.	1 : 8000.	1 : 10,000.	1 : 12,000.	1 : 15,000.	1 : 20,000
Original strain	+++	++	++	++	+	+	—	—	—	—	—
Variant . . .	++++	++++	++++	++++	++++	++++	++++	++++	+++	++	—

Reading after 4 hours at 37° C. Emulsions of equal density.

The question, of course, arises as to whether variations in agglutinability might be dependent on morphological differences, *e.g.* the presence of a glutinous capsule. In the first experiments the most agglutinable variant was the A type (large, thin, non-viscous colonies), the least agglutinable the C type (large, thick, non-viscous colonies), while the original strain (large, thick, viscous colonies) and the B type (small, thick, viscous colonies) represented intermediate degrees of agglutinability. No conclusions can be drawn to show that agglutinability is influenced by the presence of a glutinous capsule. These observations are, however, of special interest in showing how the serological characters of a particular organism, interpreted by the usual methods, may vary under certain conditions.

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THE VALUE OF SIMULTANEOUS TESTING FOR THE WASSERMANN REACTION, WITH TWO DIFFERENT ANTIGENS AND THE "ICE-BOX METHOD."

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The failure of the Wassermann reaction to detect even under optimum conditions a proportion of syphilitic cases, especially those in the early primary, tertiary and latent stages, undoubtedly represents a practical limitation which detracts considerably from the diagnostic application of the test. While the sera of many such cases show no actual difference in the Wassermann test from normal sera, it must be recognised that a weakly reacting serum may, under certain conditions, escape recognition and yield a negative result. Variations in deviability of complement may be responsible for quantitative differences in Wassermann effects, and a serum which is weakly positive with one specimen of complement may react negatively with a complement of lesser deviability. Apart from this factor, the Wassermann reaction is subject to other influences, and any procedure which adds to the sensitiveness of the test without eliciting non-specific positive results must be regarded as of the greatest practical value for the detection of sera which are likely to yield even under suitable conditions only a weak reaction.

SIMULTANEOUS TESTING WITH TWO ANTIGENS.

That the results depend among other factors on the type of antigen used has been indicated by the observations of many workers on this subject, and while an alcoholic heart extract *plus* cholesterol has proved highly satisfactory and has been much advocated, it has been shown by Browning and Kennaway (1) that the greater sensitiveness of this antigen is by no means a constant, and that with certain sera a liver lecithin *plus* cholesterol antigen may elicit positive results when the heart extract is inactive in antigenic property. Thus, if only one of these antigens is used a proportion of weakly positive reactions may be missed. We have therefore applied in a series of cases the routine procedure advocated by Browning and Kennaway, in which each serum was tested simultaneously with (*a*) liver lecithin *plus* cholesterol antigen and (*b*) heart extract *plus* cholesterol antigen, using a fixed quantity of serum, and ascertaining in each case the degree of complement fixation by testing with varying doses of complement.

Method of carrying out the Reaction.

The antigens.—(*a*) The liver lecithin *plus* cholesterol antigen was prepared according to the method of Browning, Cruickshank and Mackenzie (2); (*b*) the heart extract was obtained from sheep's heart minced, and finely ground with sand, and then extracted for four days with absolute ethyl alcohol (20 grm. heart to 100 c.c. alcohol); the extract was saturated with cholesterol (about 0·4 per cent.).

The patient's serum was heated at 57° C. for half an hour, according to the usual procedure.

The hæmolytic system was ox's red corpuscles (5 per cent. suspension of blood) with a rabbit *versus* ox immune body, and guinea-pig's serum was used as complement.

Both antigens were used for the test in a 1 : 12 dilution made up in the form of a turbid suspension by slow admixture of the alcoholic solution with the diluent (0·85 per cent. NaCl).

The M.H.D. of the complement for 0·5 c.c. of the sensitised blood suspension was estimated by preliminary test.

The amounts of the different reagents were as follows :

	Test.			Antigen Control.		Serum Control.	
	1.	2.	3.	1.	2.	1.	2.
Antigen 1 : 12	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	Saline 0·5 c.c. 0·85 %	0·5 c.c.
Serum	0·05 c.c.	0·05 c.c.	0·05 c.c.	—	—	0·05 c.c.	0·05 c.c.
Complement	4 M.H.D.	8 M.H.D.	14 M.H.D.	2 M.H.D.	4 M.H.D.	2 M.H.D.	4 M.H.D.

The tubes were incubated for one and a half hours at 37° C., and then 0·5 c.c. of the sensitised blood suspension was added to each. The results were read after one hour's incubation at 37° C. The dilution and amounts of the antigens were determined by a large series of comparative tests with several known negative and a number of positive sera. According to Ruediger (3) higher dilutions of antigen may yield more marked reactions than lower dilutions; we have not found this to be the case in our experiments, in which dilutions ranging from 1 : 6 to 1 : 500 were tested, and therefore the largest amount of antigen which constantly failed to elicit non-specific reactions or a marked anti-complementary effect by itself was selected for routine testing. These tests were carried out with a number of different specimens of guinea-pig's complement, and it was generally found that the optimum amount of both antigens thus determined was 0·5 c.c. of a 1 : 12 dilution (using 0·05 c.c. of patient's serum).

It was noted in the case of certain sera (from treated cases) that amounts of serum less than 0·05 c.c. failed to yield a positive result; this amount of serum never elicited non-specific reactions. Larger quantities in some cases showed marked anti-complementary effects, so that this amount was selected as a likely optimum.

On testing by this method a number of known negative and weakly positive sera (from treated syphilitic cases which had previously yielded a strongly positive reaction) with different specimens of guinea-pig's complement, which of course varies considerably in deviability, it was found that in the case of both antigens with negative sera 4 M.H.D. (tube 1) was the smallest amount of complement which constantly produced "almost complete" or "complete" lysis, provided the antigen and serum were not excessively inhibitory by themselves as shown by the respective controls. At the same time the weakly positive sera with one or both antigens absorbed this amount of complement, and no lysis or only a trace of lysis occurred in the first tube. Thus the occurrence of complete or almost complete inhibition of lysis in the first tube was regarded as a criterion of a positive reaction, provided that negative control sera (which were always included in each series of tests) showed complete or almost complete lysis in this tube (see tables). Of course, in the majority of positive reactions the deviation is more marked.

A series of 300 sera were tested with this technique and using two antigens, with the following results:

1. *Negative* with both antigens	138	
2. *Positive* with both antigens, and showing an equal degree of complement-deviation with both	81	50 % of the total positive reactions.
3. *Positive* with both antigens, but showing more marked complement-deviation with the heart cholesterol antigen	34	20·98 % ditto.
4. *Positive* with both antigens, but showing more marked complement-deviation with the lecithin cholesterol antigen	17	10·49 % ditto.
		81·47 % ditto.
5. *Positive* with the lecithin cholesterol antigen, but *negative* with the heart cholesterol antigen	11	6·79 % ditto.
6. *Positive* with the heart cholesterol antigen, but *negative* with the lecithin cholesterol antigen	19	11·72 % ditto.
		18·51 % ditto.

Note.—"Positive" includes also "weak positive" reactions.

Of the positive results 81·4 per cent. yielded definite reactions with both antigens; 50 per cent. showed an equal degree of complement-deviation with the two antigens, while 20·9 per cent. gave a more marked result with the heart antigen and 10·4 per cent. a more marked effect with the liver lecithin antigen. Thus the heart antigen was usually the more sensitive, but not invariably so; 11·7 per cent. gave a positive reaction with this antigen, while the same sera reacted negatively with the lecithin antigen; on the other hand 6·7 per cent. reacted positively with the lecithin antigen while the heart extract gave a negative result. About 18·5 per cent. of all the positive results belonged to this category in which a positive result was obtained with one antigen while a negative effect occurred with the other. The 11 cases belonging to class 5 comprised 6 early primary cases, 4 treated cases, and 1 case of aortic aneurysm. The cases in class 6 were: 7 early primary cases, 11 cases which had received antisyphilitic treatment, and 1 case of iritis with a syphilitic history. Accordingly it was possible to detect cases in the early primary stage by using the two antigens, when one might fail to elicit a positive result if only a single antigen were used. It has been suggested by Walker and Swift (4) that the value of the heart antigen is particularly marked in testing treated cases, and while this is evident, the liver lecithin cholesterol antigen is also capable of detecting a certain proportion of such cases which do not react positively with the heart antigen.

The Table shows some of these results. In series 1, while three sera reacted positively with the heart antigen, one serum (C) was positive only with the liver antigen. It was sometimes observed that with one complement all the positive sera reacted more strongly with one antigen than the other (series 2), but with the same specimen of complement "converse" effects were frequently noted, as shown in series 1 and 3.

Apart from the value of multiple antigens in eliciting weakly positive reactions, it frequently happens that one antigen yields a more strongly positive reaction than the other and renders the result more conclusive, and even if the results are identical the use of a second antigen supplies a valuable confirmatory test. The method is particularly valuable in primary cases and those which are being tested as a guide to treatment.

THE ICE-BOX METHOD.

In a recent report on this method of performing the Wassermann test, Fairley and Sullivan (5) indicated that the sensitiveness of the reaction was distinctly increased by allowing the interaction of antigen, serum and complement to occur at 8° C. for a period of six hours, as compared with the results of incubating at 37° C. by the usual method. They supposed that the value of the procedure depends essentially on the increased time allowed for complement-fixation to occur, and showed that complement-fixation takes place as readily at low temperatures as at 37° C., and that it is possible to expose complement for a long period at 8° C. without deterioration in its hæmolytic value. As this procedure seemed to suggest a possible method of increasing the sensitiveness of the reaction, we have carried out a series of 102 tests in which the effect of incubation at 37° C. for one and a half hours was compared with that of allowing the interaction to occur at 8° C. for six hours, using the two antigens referred to above in both cases. Of the 102 sera tested in this way

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Explanation of symbols: L = Liver lecithin *plus* cholesterol. H = Heart extract *plus* cholesterol. c = complete lysis. jc = just complete. alc = almost complete. mk = marked. dst = distinct. tr = trace. o = no lysis.

Note.—The complement M.H.D. in Series 1 and 3 = 0·004 c.c.; in Series 2, 0·006 c.c.

The serum controls gave complete or just complete lysis with two doses of complement in every case. The antigen controls with two doses of complement gave just complete lysis in each series, except in the case of heart extract *plus* cholesterol in Series 1, which gave almost complete lysis. The tests of the positive and negative control sera have been omitted from Series 2 and 3.

SERIES 1.

	Antigen.	Test.			Result.
Tube	—	1	2	3	—
Doses of complement	—	4	8	14	—
Negative control serum	L	jc	c	c	Negative.
	H	jc	c	c	,,
Positive control serum	L	o	o	tr	Marked positive.
	H	o	o	tr	,, ,,
Serum A: Primary sore	L	alc	c	c	Negative.
	H	o	tr	jc	Positive.
Serum B: Treated case	L	alc	c	c	Negative.
	H	tr	alc	c	Weak positive.
Serum C: Treated case	L	tr	alc	c	,, ,,
	H	jc	c	c	Negative.
Serum D: Treated case	L	alc	c	c	,,
	H	tr	alc	c	Weak positive.

SERIES 2.

	Antigen.	Test.			Result.
Serum A: Primary sore	L	c	c	c	Negative.
	H	tr	alc	c	Weak positive.
Serum B: Iritis	L	c	c	c	Negative.
	H	tr	alc	c	Weak positive.
Serum C: Primary sore	L	alc	c	c	Negative.
	H	o	tr	dst	Positive.
Serum D: Secondary syphilis	L	o	tr	mk	,,
	H	o	o	o	Marked positive.
Serum E: Primary sore	L	o	dst	c	Positive.
	H	o	o	dst	Marked positive.
Serum F: Tabes dorsalis	L	tr	c	c	Weak positive.
	H	o	tr	c	Positive.

SERIES 3.

	Antigen.	Test.			Result.
Serum A: Treated case	L	tr	jc	c	Weak positive.
	H	jc	c	c	Negative.
Serum B: Treated case	L	c	c	c	,,
	H	tr	dst	c	Positive.
Serum C: Primary sore	L	c	c	c	Negative.
	H	tr	alc	c	Weak positive.
Serum D: Primary sore	L	alc	jc	c	Negative.
	H	o	tr	c	Positive.
Serum E: Treated case	L	tr	mk	c	Weak positive.
	H	alc	c	c	Negative.
Serum F: Known positive	L	o	o	mk	Positive.
	H	o	alc	c	Weak positive.
Serum G: Known positive	L	o	alc	c	,, ,,
	H	o	o	dst	Positive.

50 reacted negatively and 52 positively by both methods, and there was no qualitative divergence of results. As regards the comparative degree of complement-deviation by the two methods the results were as follows:

Complement-fixation by the Ice-box Method as compared with that at 37°C. by the Usual Method.

Heart antigen.	Liver lecithin antigen.	
More marked . . .	More marked . . .	3 cases.
Less marked . . .	Less marked . . .	5 ,,
Equal (approximately) .	More marked . . .	1 case.
More marked . . .	Equal (approximately) .	3 cases.
Equal (approximately) .	Less marked . . .	3 ,,
Less marked . . .	Equal (approximately) .	1 case.
Equal (approximately) .	Equal (approximately) .	36 cases.

It seems doubtful from these results if this method yields any advantages as a substitute for the original method, and it has the disadvantage of the long period required for carrying out the complete test. In certain suspicious cases, however, yielding a negative result, it might be applied for purposes of confirmation. It is also of interest to note the differences elicited between the action of the two antigens in these comparative tests.

CONCLUSIONS.

(1) The sensitiveness of the Wassermann reaction as here described can be increased, especially in early primary and treated cases, by testing simultaneously with a liver lecithin *plus* cholesterol antigen and a heart extract *plus* cholesterol antigen, *i. e.* a proportion of weakly positive reactions can be detected in this way which would escape notice if only one of these antigens were used.

(2) The ice-box modification as applied to the method described does not confer any appreciable advantage over the usual procedure of incubating the mixture of antigen, serum and complement at 37°C. for one and a half hours.

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VARIATIONS IN THE BENZIDINE PEROXIDASE REACTION DEPENDING ON FIXATIVE, PHYSIOLOGICAL ACTIVITY AND TYPE OF ANIMAL.

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In 1909, Schultze (¹) discovered that when films of blood were immersed in solutions of α-naphthol and dimethylparaphenylenediamine these substances were oxidised by contact with cells of myelogenous origin, as was shown by the development within them of coloured granules. Cells of lymphatic origin, lacking this oxidising property, remained uncoloured under the same conditions. The demonstration that the difference in the composition of the cells of these two series was sufficiently marked to influence either favourably or unfavourably the oxidation of adsorption complexes of phenolic dye substances within the leucocytes led to a further study of many phenol derivatives similar to α-naphthol. Among the more recently investigated of these phenols is benzidine, whose first employment in the microchemistry of the blood is accredited to Fischel.(²) However, the use of this base as a microchemical agent for the demonstration of the so-called peroxidases has not always yielded uniformly positive results in the hands of the investigators who have reported upon its use. Among the undesirable effects which have been obtained are staining of elements other than the myelogenous cells, the transient character of the colour formed, and the difficulty in finding a suitable counterstain. Indeed, so unsatisfactory did Fischel (²) find benzidine that he was led to substitute for the base the benzidine sodium monosulphate. However, Fiessinger and Rudowska (³), Loele (⁴) and Graham (⁵) each successfully developed with benzidine (base) a technique, by means of which the cells of the myelogenous series could be readily differentiated from the lymphocytes though the colour-formation was not identical in the different methods used. Thus Fiessinger and Rudowska (³), after immersing blood-films in a 2 per cent. solution of benzidine in absolute alcohol and then exposing them to solutions of hydrogen peroxide, obtained blue granules. Graham (⁵), treating blood films with a 0·5 per cent. solution of benzidine in 75 per cent. alcohol to which was added 0·2 per cent. hydrogen peroxide, found brown granules in neutrophiles and eosinophiles.

Alcoholic benzidine to which hydrogen peroxide has been added is a reagent in which a series of oxidation changes may be induced by exceedingly slight modifications in the solvent. The fact that different colours may be produced in the benzidine taken up by the polymorphonuclear leucocytes suggests that these colour differences in the cells may be due either to variations in the composition or in the physico-chemical state of these blood elements.

Our attention was first directed to such possible variations in the course of some experiments in which benzidine was employed as one of the methods of demonstrating the peroxidase content of the leucocytes in blood from experimental guinea-pigs. In using the Graham solution outlined above and carefully following the technique recommended we failed to duplicate the blood-picture described by the author. Only a small percentage of the polymorphonuclear leucocytes of the guinea-pig contained yellow granules. Further investigation revealed the fact that an alcoholic solution of benzidine, both with and without the addition of hydrogen peroxide, would not only reveal differences in the peroxidase content between cells of myelogenous and lymphatic origin, but by slightly modifying the concentration of the various constituents of the solution, variations in the intrinsic composition of the neutrophilic leucocytes from different animals could further be distinguished. Only neutrophiles and eosinophiles of human blood give the results reported by Graham. The benzidine reagent recommended by that author is not specific for all myelogenous cells from the blood of lower animals. (We have not had the opportunity of comparing the benzidine reaction in leucocytes of human blood with that of the white blood-cells from the other families of the Anthropoidea more nearly related to man. It would seem probable that blood elements from some of the genera of the Simiidæ might give reactions similar to those of man.)

Besides differences in composition of cells peculiar to various animals, physiological activity and fixation were modifying factors which contributed to variations in the leucocyte reaction. Results of the studies dealing with these various factors are outlined in the following pages.

The solution used in the first series of experiments was as nearly as possible that described by Graham, and consisted, as already stated, of a 0·5 per cent. solution of benzidine in 75 per cent. alcohol, to which was added 0·2 per cent. hydrogen peroxide. In the early experiments the hydrogen peroxide and the benzidine were added to the alcohol at the time of preparation and the solution was then allowed to stand for forty-eight hours or longer. In the later experiments, and especially in the study of pus, it was found more convenient to make up a stock solution of alcoholic benzidine and to add the hydrogen peroxide immediately before the staining of the preparations. The films, after being thoroughly washed in distilled water, were counterstained with a saturated solution of thionin in 75 per cent. alcohol, to every 10 c.c. of which was added 2 drops of a 2 per cent. solution of sodium carbonate immediately before using. The films were then mounted in 50 per cent. aqueous glycerine.

The benzidine consisted of two Merck preparations. One was a white crystalline salt and the other was a fine greyish-mauve powder. No appreciable difference could be distinguished between the results obtained by either substance. We did not note that the mauve powder gave a more diffuse reaction as described by Graham. The hydrogen peroxide was U.S.P., neutral in reaction, and contained $\frac{1}{5}$ gr. of acetanelid to the fluid ounce. While the reaction could be readily obtained with freshly prepared benzidine solutions the formation of colour was somewhat accelerated when this had been prepared one or two days previously, during which time it acquired a brown colour due to oxidative changes. In order to determine the time of immersion required to give the optimal results the time of staining was varied from five minutes to seven hours, but as in practically every instance a well-marked colour was developed in fifteen minutes this was arbitrarily chosen as the exposure time for all films studied. Only blood-cells were studied. To compare the benzidine reaction in blood of different animals unfixed films were used and these were dried in air. They must be thin and evenly spread, as the colour which develops depends somewhat on the thickness of the film. Freshly made films were used, although no appreciable difference in the intensity of the colour could be detected if these were kept for two or three weeks provided they were not exposed to the action of light. As it was noted that fixation was not a negligible factor in the development of colour the effect of treatment with 5 per cent. Schering's formalin and with absolute alcohol previous to staining with benzidine was studied. The results are noted in the appended tables.

Blood was taken from human patients and from the following animals: white rat, white mouse, guinea-pig, dog, rabbit and hen.

When one treats films of blood from the above animals with the Graham solution of benzidine and compares them with similarly treated films of human blood, one observes, in agreement with the findings of the majority of the other workers in this field, that the lymphocytes in all of the specimens are invariably negative. The reaction in the cells of the myelogenous series is not uniformly positive, but varies with the type of animal and with the varying concentrations of alcohol and of hydrogen peroxide used. The granules of all of the human polymorphonuclear leucocytes are invariably stained a yellowish-brown, while in those of the lower animals these either remain completely negative or only a small percentage stain. In five guinea-pigs, counts of the polymorphonuclear leucocytes were made, and the estimated percentages of positive cells among these were 8·7, 10·4, 11·6, 15 and 20·5. These figures vary within fairly wide limits, and while these variations are no doubt partly due to the method of preparing the films, they can be largely accounted for by differences in the blood of the individual animals. Mouse and rat showed similar variation. In some of the rabbits studied, and especially the younger ones, the yellow colour in the granules was very pale at the end of fifteen minutes' staining, and a well-defined reaction could be obtained only by prolonging the immersion to thirty or forty minutes. In all of these animals, if the hydrogen peroxide was increased to 1 per cent., all of the cells except an occasional polymorphonuclear leucocyte were negative. However, if the same benzidine solution free from hydrogen peroxide was used then the percentage of positively reacting cells rose to a 100. Adding hydrogen peroxide in progressively increasing quantity decreased the number of positively reacting cells, which became almost completely negative at 1 per cent. In the few cells showing yellow granules at this concentration the tendency to retain this colour is very marked. Colour-formation in these cells is only inhibited by increasing the hydrogen peroxide to a high concentration. Both the size of this cell and its contained granules are larger than normal. A greenish cast was often observed in the negatively reacting cells.

The blood of the hen was interesting, since leucocytes containing the rod-shaped granules were negative not only in solutions free from hydrogen peroxide, but also in those to which 0·2 and 1·0 per cent. hydrogen peroxide had been added. In the first solution all of the polymorphonuclear leucocytes containing the round granules were positive. At 0·2 per cent. the number had decreased to about a half, and at 1 per cent. all of the cells with round, coloured granules were practically absent. The thrombocytes were negative in all three solutions. In the erythrocytes a definite yellow reaction occurred when 1 per cent. hydrogen peroxide was present in the reagent, and when the concentration of this substance reached 1·2 per cent. brown granules were found lying around the nucleus.

If the slides already described were re-examined four or five weeks after they were originally stained, the nuclear thionin staining had disappeared and the brown "peroxidase" colour was much more evident. This is probably due to the continued oxidation of the benzidine compounds which have been adsorbed by the cell. These preparations are readily re-stained with thionin.

As the percentage of hydrogen peroxide is increased the yellow reaction is also observed in the erythrocytes, but it was not found in a concentration below 0·4 per cent. It also occurs by prolonging the time of immersion, thus confirming Graham's (5) observation. Only an occasional cell exhibiting granules stained with thionin was found in any of the preparations.

While minor differences exist between the blood from different orders, the most marked difference was found between man and the order next below. Only in man and dog have these variations been studied in detail. The concentrations of alcohol used in these studies were 45, 75, 85, 90, 95 and absolute. In all except the first the concentration of benzidine was 0·5 per cent. In 45 per cent. alcohol benzidine is not soluble to this extent and a saturated solution was therefore used. Smears were stained in each of the following solutions, viz. one free from alcohol, and the other two containing hydrogen peroxide in the amounts of 0·2 and 1·0 per cent. Films made at the same time were not only dried in air but also fixed in 5 per cent. formalin and absolute alcohol, and these three series of slides were then subjected to the same technique. The results have been arranged in Tables I and II (pages 230–231).

There are several points of interest in these tables. Perhaps the most striking feature is the fact that the human smears, both with and without fixation, are not coloured by the benzidine solutions unless hydrogen peroxide has been added. No colour is developed in human blood-cells in alcoholic benzidine. It is also interesting to note that unfixed human cells respond much less readily to the action of benzidine than the unfixed cells from the blood of the lower animals.

Formalin treatment previous to staining enhances the effect of the benzidine in all the bloods examined when the higher concentrations of alcohol are used. Thus the highest concentration of alcoholic benzidine in which films of untreated human blood stain is 85 per cent., while following formalin fixation positive results can be obtained with 95 per cent. alcoholic solutions. However, when 75 and 45 per cent. alcohol are employed as solvents the efficiency of the benzidine as a staining reagent is diminished. The intensity of the resulting reaction when hydrogen peroxide is used depends largely upon the strength of the alcohol and consequently upon the degree of dissociation of the benzidine.

In the lower animals the characteristic yellow staining in leucocytes can be readily obtained with benzidine solutions which are free from hydrogen peroxide. With fixed preparations in the 75 and 85 per cent. alcoholic solutions there is a marked tendency for the formation of dark blue acicular crystals to occur. While the crystals appear to arise from material which has been extruded from the cell this is difficult to decide definitely. The loss of the granular character of the yellow-brown reaction may be met with not uncommonly in the lower concentrations of alcohol after formalin fixation, and in these cases the colour is reduced in intensity and assumes a pale yellow cast. The colour may be found in all shades, from a pale yellow to a deep brown. This coloured material, except where the granular aspect is lost, lies on the periphery of the granule, and undoubtedly is of the character of an adsorption compound similar to that described by us (8) and found with aqueous solutions of α-naphthol and paraphenylenediamine.

With negative results the cells frequently possess a very pale greenish-yellow cast. The action of formalin fixation, as is to be observed from the tabulated results, differs somewhat from that of absolute alcohol. With the latter less divergence from the colour picture obtained in unfixed films is observed. As previously indicated, only in thin, evenly-spread films are uniform clear-cut results found. Where the cells lie closely packed the staining may be diffuse and the colour then varies considerably.

TABLE I.—*Blood from Human Patient.*

0·5 per cent. benzidine is contained in each of the following solutions.	Films unfixed, dried in air.	Films fixed in 5 per cent. formalin.	Films fixed in absolute alcohol.
Absolute alcohol	Negative except for 1 or 2 cells	Negative except for 1 or 2 cells	Negative except for 1 or 2 cells.
Ditto + 0·2 per cent. H_2O_2	Ditto	,, ,, ,,	Ditto.
,, + 1·0 per cent. H_2O_2	,,	,, ,, ,,	,,
95 per cent. alcohol	Negative except for 1 or 2 cells	All cells contain yellow granules { in large cells colour ++++ ,, small ,, ,, +++ }	Negative except for 1 or 2 cells.
Ditto + 0·2 per cent. H_2O_2	Ditto	Ditto { ,, large ,, ,, +++ ,, small ,, ,, ++ }	Ditto.
,, + 1·0 per cent. H_2O_2	,,	,, { ,, large ,, ,, ++ ,, small ,, ,, + }	,,
90 per cent. alcohol	Negative except for 1 or 2 cells	All cells contain yellow granules and cells are surrounded by deep blue acicular crystals	Negative except for 1 or 2 cells.
Ditto + 0·2 per cent. H_2O_2	Ditto	All cells contain yellow granules less intense in colour and granules present	50 per cent. of cells contain yellow granules.
,, + 1·0 per cent. H_2O_2	,,	All cells contain pale granules and deep blue acicular crystals surround the cells	5 per cent. of cells contain yellow granules.
85 per cent. alcohol	Negative except for 1 or 2 cells	All cells negative. Few blue crystals surround the cells	Negative except for 1 or 2 cells.
Ditto + 0·2 per cent. H_2O_2	All cells contain yellow-brown granules	All cells contain pale yellow granules. Occasional blue crystals lying on periphery of cell	15–20 per cent. of cells contain yellow granules.
,, + 1·0 per cent. H_2O_2	Ditto	All cells positive with both yellow- and blue-coloured granules	Ditto, but less intense colour
75 per cent alcohol	Negative except for 1 or 2 cells	All cells coloured yellow. Granules cannot be distinguished	All cells contain faint yellow granules.
Ditto + 0·2 per cent. H_2O_2	All cells contain yellow-brown granules	All cells coloured pale yellow. Granules cannot be distinguished	Negative except for 1 or 2 cells.
,, + 1·0 per cent. H_2O_2	Ditto	Ditto	Ditto.
Solutions each containing benzidine to saturation.			
45 per cent. alcohol	Negative except for 1 or 2 cells	All cells contain yellow-brown granules	All cells contain yellow-brown granules.
Ditto + 0·2 per cent. H_2O_2	All cells contain yellow-brown granules	Ditto	Ditto.
,, + 1·0 per cent. H_2O_2	All cells contain yellow granules	Ditto, but less intense in colour	All cells contain yellow granules.

All cells referred to in the above table are polymorphonuclear leucocytes.

TABLE II.—*Blood from Dog.*

0·5 per cent. benzidine is contained in each of the following solutions.	Films unfixed, dried in air.	Films fixed in 5 per cent. formalin.	Films fixed in absolute alcohol.
Absolute alcohol	Negative except for 1 or 2 cells	12–15 per cent. of cells contain yellow-brown granules	12–15 per cent. of cells contain yellow-brown granules.
Ditto + 0·2 per cent. H_2O_2	Ditto	Ditto	Ditto.
„ + 1·0 per cent. H_2O_2	„	„	3 per cent. of cells contain yellow granules.
95 per cent. alcohol	12–15 per cent. of cells contain yellow-brown granules	12–15 per cent. of cells with deep yellow-brown granules	12–15 per cent. of cells with deep yellow-brown granules.
Ditto + 0·2 per cent. H_2O_2	Ditto (paler)	12–15 per cent. of cells with yellow-brown granules	All cells contain yellow-brown granules.
„ + 1·0 per cent. H_2O_2	Negative except for 1 or 2 cells	12–15 per cent. of cells with yellow granules	All cells contain deep yellow-brown granules.
90 per cent. alcohol	12–15 per cent. of cells contain yellow-brown granules	All cells contain yellow-brown granules	All cells contain yellow-brown granules.
Ditto + 0·2 per cent. H_2O_2	12–15 per cent. of cells contain yellow granules	Ditto. Cells are surrounded by blue crystals	All cells contain pale yellow-brown granules.
„ + 1·0 per cent. H_2O_2	12–15 per cent. of cells contain faint yellow granules	Reaction similar to above, but colour more intense	All cells contain yellow granules.
85 per cent. alcohol	All cells contain yellow-brown granules	All cells contain yellow-brown granules.	All cells contain yellow-brown granules.
Ditto + 0·2 per cent. H_2O_2	50 per cent. of cells contain yellow-brown granules	All cells contain brown granules. Cells appear fissured and surrounded by blue crystals	Ditto.
„ + 1·0 per cent. H_2O_2	All cells negative	Reaction similar, but brown colour more marked	12–15 per cent. of cells contain yellow-brown granules.
75 per cent. alcohol	All cells contain yellow-brown granules	All cells contain brown granules	All cells contain yellow-brown granules.
Ditto + 0·2 per cent. H_2O_2	10–15 per cent. of cells contain yellow-brown granules	All cells contain yellow-brown granules	12–15 per cent. of cells contain yellow granules.
„ + 1·0 per cent. H_2O_2	All cells negative	12–15 per cent. of cells contain yellow-brown granules	Negative except for 1 or 2 cells.
Solutions each containing benzidine to saturation.			
45 per cent. alcohol	All cells contain brown granules	Negative except for 1 or 2 cells	All cells contain yellow-brown granules.
Ditto + 0·2 per cent. H_2O_2	All cells contain yellow-brown granules	All cells contain yellow-brown granules	Ditto.
„ + 1·0 per cent. H_2O_2	10–15 per cent. of cells (large in size) contain yellow granules	12–15 per cent. of cells contain yellow-brown granules	„

All cells referred to in the above table are polymorphonuclear leucocytes.

When smears treated with benzidine are compared with those obtained by other oxidase and peroxidase reagents, such as the Graham α-naphthol-pyronin and the various modifications of the α-naphthol and paraphenylenediamine, the above-discussed variations are not found. In benzidine, therefore, we possess a microchemical reagent which is more sensitive to changes in the composition of the leucocyte than in these other methods. We were able to compare the blood-films from one case of acute myelogenous leukæmia with normal human blood-cells. In the former there was no appreciable difference in the staining from those of the normal.

In a case of lymphatic leukæmia, under none of the conditions of the experiments indicated in Table I did we obtain staining of the lymphocytes.

Since alcoholic benzidine is a sufficiently sensitive reagent to indicate microchemical differences in myelogenous cells of various animals and under varying conditions, it was thought not unlikely that evidence of the grosser changes known to occur in pus-cells might also be revealed by this reagent. With this object in view, preparations of human pus were studied in a manner similar to that of normal blood except that fixation with absolute alcohol was omitted. Smears were studied both with and without counterstain. With pus, as might be expected, different results depending upon the severity and kind of infection and the amount of change which has taken place in the cells can be obtained. Where cells are very much degenerated and contain many bacteria the whole film is negative. Our most instructive results were obtained with pus taken from three cases of empyema of pleura and a case of suppurative arthritis of the knee-joint. As the results obtained with all of these samples of pus resemble one another closely only one will be described. The details of this are included in Table III.

All four samples of pus were removed by aspiration and studied as soon after removal as possible. When kept in the refrigerator fairly concordant results could be obtained for two or three days, but the preparations slowly degenerated and the positive reaction in the granules gradually changed to a negative. If the sample is allowed to stand at room temperature for three or four days it is converted into a negatively reacting specimen. Not only does the pus in the test-tube gradually lose its affinity for the benzidine, but the unstained as well as the stained films react negatively with the lapse of time. In the above four cases with the various concentrations of alcohol, a series of colours ranging from deep brown through yellow and yellow-green to blue were observed in the granules following immersion in benzidine solution. The blue colour is very sharply defined and is most commonly found with concentrations of alcohol of 85 and 90 per cent., though it may even be very distinct in the solutions made up with alcohols of 95 and 75 per cent. In the two latter concentrations, cells containing mixtures of yellow and blue granules are common, but many cells are filled with only the blue or the yellow granules. This blue colour found in pus is identical with that developing in granules of normal cells of both human and dog's blood to which acid has been added, and indeed if a trace of acid from the cleaning solution accidentally remains on the slide on which the pus smear is spread the blue colour in the granules appears immediately. The appearance of this blue colour is very much like that which is figured and described by Graham (5) in cells following treatment with weak acids. The four samples of pus studied were neutral to litmus. Formalin fixation prevents the formation of this blue colour, which appears to be much less resistant than the yellow. Variations in the intensity of the yellow colour

TABLE III.—*Pus from Empyema of Pleural Cavity.*

0·5 per cent. benzidine is contained in each of the following solutions.	Films unfixed, dried in air.	Films fixed in 5 per cent. formalin.
Absolute alcohol	Negative	Negative.
Ditto + 0·2 per cent. H_2O_2	Ditto	Negative except for bluish colour in nuclei.
„ + 1·0 per cent. H_2O_2	„	Negative.
95 per cent. alcohol	Negative	Negative.
Ditto + 0·2 per cent. H_2O_2	Cells contain both yellow and blue granules	Ditto.
„ + 1·0 per cent. H_2O_2	All cells contain blue granules	Negative except for pale-blue colour in nuclei developing immediately on immersion and lasting only a few minutes.
90 per cent. alcohol	Negative	Negative.
Ditto + 0·2 per cent. H_2O_2	All cells contain blue granules	All cells contain brown granules.
„ + 1·0 per cent. H_2O_2	Ditto	All cells contain deep brown granules.
85 per cent. alcohol	Negative	Negative.
Ditto + 0·2 per cent. H_2O_2	All cells contain blue granules	All cells coloured yellow. Granules not distinct.
„ + 1·0 per cent. H_2O_2	Ditto	All cells coloured yellow. Occasional brown granules distinct, but reaction tending to become diffuse.
75 per cent. alcohol	Negative	Negative.
Ditto + 0·2 per cent. H_2O_2	Cells contain both yellow and blue granules	All cells contain yellow-brown granules. Transient blueing of nuclei.
„ + 1·0 per cent. H_2O_2	Most of the cells contain blue granules, but a few show both yellow and blue granules	Ditto.
Solutions each containing benzidine to saturation.		
45 per cent. alcohol	Negative	Negative.
Ditto + 0·2 per cent. H_2O_2	All cells contain brown granules	All cells contain brown granules. Transient blueing of nuclei.
„ + 1·0 per cent. H_2O_2	All cells contain deep brown granules	Ditto.

from a very faint yellow to a dark brown occur. In formalin preparations the maximum brown colour appeared with 45 per cent. alcohol *plus* 0·2 per cent. hydrogen peroxide.

After formalin fixation, with the solutions made up with 90 and 95 per cent. alcohol and containing hydrogen peroxide, a faint blue colour develops in the nuclei immediately upon immersion. This colour is very evanescent, and disappears if the film is allowed to remain in the reagent for more than one or two minutes. When the preparations are counterstained it is completely obscured. If absolute instead of 90 or 95 per cent. alcohol is used as a solvent the blue colour is more pronounced, but it is then only observed when the hydrogen peroxide is present in a concentration of 0·2 per cent.

Pus from abscesses and discharging sinuses invariably gives a negative reaction, in which case the cells show considerable degeneration.

DISCUSSION.

A glance over the results recorded in Tables I and II will suffice to convince one that widely differing effects may be obtained with benzidine as a microchemical reagent. By varying slightly the alcohol or the hydrogen peroxide of the reagent the same film may be transformed from a positive to a negative. Films from different types of animals do not always give identical pictures when immersed in the same solution.

Because the benzidine reagent responds by colour changes to such slight variations in its composition, its usefulness as a means of differentiation of cells of myelogeneous origin is lessened unless these variations are controlled. It does, however, offer possibilities of being adapted as a microchemical method for studying minor differences among these cells.

That minor differences exist between the red blood-cells of various animals has already been well established by the separation of different crystalline forms of hæmoglobin and by the finer serological technique. Methods for demonstrating the existence of analogous differences in leucocytes are at present not available, but that similar variations do obtain is indicated by results obtained by alcoholic solutions of benzidine. While the latter lack the precision and delicacy which marks the serological technique, that further modifications may show still finer differences in the composition of these white blood-cells is suggested by Graham (7) in his work on basophiles. As has been already indicated, the addition of hydrogen peroxide in minute quantities so modifies the reaction of the reagent that it will differentiate the human cells of myelogenous origin from those of many of the lower animals.

While it is probable that these variations are due in large part to actual differences in the cellular composition, it is not to be overlooked that part of the variation may also be due to physico-chemical changes induced by slight differences in the media surrounding the cells. Thus, for example, by exposure of blood-films to traces of acid, colour formation in cell granules analogous to that found in samples of pus may be developed. While the degree of acidity required to give this reaction is certainly never reached in normal blood *in vivo*, the quantitative as well as qualitative differences in the salt content of blood-plasma of various organisms will exert a similar (or opposite) action, though in a smaller degree.

That variations in the hydrogen-ion content of the environment operate *in vivo* as a modifying factor is clearly demonstrated in colour results obtained in samples of pus aspirated from cavities in the body. Whether the colour-reaction depends, as has been postulated by various investigators, upon the presence within the cell of the hypothetical substances, the so-called oxidases and peroxidases, which are capable of directly transferring oxygen from the cell constituents to certain dyes, seems to be very doubtful. It would seem that, rather than looking upon these substances as chemical entities, the phenomenon of intracellular colour development might be more readily explained as being due to physico-chemical changes in tissues—a view which we have already discussed in two previous papers (8), (9).

Since all of the colours developing under the experimental conditions described in this paper are due to oxidation of the benzidine, and oxidation always results directly or indirectly from the acquisition by the oxidised body of positive electrical charge, the reaction is due fundamentally to a transfer of this charge in part or whole from the cellular constituents to the dissociated particles of benzidine which they have taken up. That the inclusion of this base by the cell is an adsorption process is strongly suggested by the observation that the benzidine taken up by the cells lies on the periphery of the granules instead of being incorporated within them—a fact which strengthens the assumption of the physico-chemical nature of oxidases and peroxidases.

SUMMARY.

(1) The Graham benzidine peroxidase reagent, consisting of a 0·5 per cent. solution of benzidine in 75 per cent. alcohol *plus* 0·2 per cent. hydrogen peroxide, produces a yellow-brown colour in the granules of myelogenous cells in human blood and is diagnostic for man.

(2) The polymorphonuclear leucocytes of blood of lower animals studied differ from those of human blood in their reaction to the Graham reagent, in that only a small percentage of them develop the characteristic yellow colour. These positively reacting cells are probably eosinophilic.

(3) In a 0·5 per cent. solution of benzidine in 75 per cent. alcohol free from hydrogen peroxide practically all of the myelogenous cells of the guinea-pig, dog, white mouse, white rat and rabbit give the characteristic coloured granular reaction. Addition of hydrogen peroxide to this solution tends to inhibit the development of the yellow colour, which disappears from all except one or two cells when the concentration of the peroxide is increased to 1 per cent.

(4) By varying the concentrations of the alcohols in the Graham reagent a series of colours ranging from deep brown through the yellows and the yellow-greens to blue may be obtained in granules of human pus aspirated from empyemic cavities. As these samples of pus age, the colour reactions are lost. Pus in which the leucocytes become more degenerated give a negative reaction.

(5) The blue reaction of pus-cells may be duplicated by exposing blood-films to weak concentrations of acid, previous to treatment with benzidine solutions.

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RESEARCHES ON DYSENTERY.

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During 1913, 1914, and 1915 I conducted a series of experiments for the Local Government Board on the nature of Ultramicroscopic Viruses, and the main results obtained were published in the *Lancet*, December 4th, 1915. In these experiments it was shown that vaccinia often contained micrococci which failed to grow on account of the presence of an unknown material which dissolved the cocci before the colonies had grown sufficiently to be visible. It was also shown that this dissolving material passed through a porcelain filter, that it was able to attack and dissolve into granules a pure culture of the micrococcus, and that from fresh cultures, the growth of which had been made transparent, it could be carried through an indefinite number of generations to fresh cultures of the micrococcus, although no visible growth of the dissolving material by itself was obtained. The activity of the material was retained for six months, but it was destroyed when heated to 60°C. for one hour. The same general results were obtained with a member of the coli-typhoid group of bacilli, and it was suggested that the difficulty often experienced in isolating certain known pathogenic micro-organisms might be due to the same cause. In cases of infantile diarrhœa similar results were obtained with some rather large bacilli, thought at the time not to belong to the dysentery-typhoid-coli group but since believed to be a variety of this group. In the paper referred to, the relation between the dissolving material and the bacterium was discussed, as was also the possibility of the dissolving material being an ultramicroscopic virus. It was also suggested that similar results would be obtained in cases of dysentery and allied conditions to be met with in the Dardanelles.

Some experiments by Dr. F. d'Herelle, presented by Prof. Roux, and published in the *Comptes Rendus, Academie des Sciences* (No. 11, 10th September, 1917), show results which confirm those given above. Dr. d'Herelle has worked chiefly on dysentery, and has obtained a filter-passing substance which dissolves up the Shiga bacillus. He states that the dissolving material can be acclimatised to act on other bacilli, and from the experiment that shows the dissolving action to start from distinct spots or areas he concludes that the substance is an ultra-microscopic virus. As I have already stated, I do not think that this point has been proved in view of the experiment in which I obtained an *apparent* spontaneous start of the dissolving action in cultures of the coccus which appeared to have remained free from the dissolving material for many generations previously. However, although this is an important point it does not affect the main experimental facts.

In the original experiments conducted at the Brown Institution some additional observations were made besides those which have already been mentioned. These were not published at the time as their exact significance had not been fully determined. It is a well-known fact that various species of the dysentery-typhoid-coli group when apparently in pure growth produce forms that are considerably longer than the typical short forms, and variations in the size and shape of individual bacilli in a pure culture have often been described. Recently Hort and others have carried out a considerable number of experiments with the object of throwing light on the significance of these different forms.

In certain experiments dealing with the dissolving material I observed these long forms in greater number than usual, and it was thought that they might in some way be connected with the dissolving material which had also been found associated with this group. On the other hand there was the obvious possibility that they might be distinct but symbiotic bacilli, or again they might represent a stage in the life history of the bacillus. It was also thought possible that they might be special forms with special work to do like bees in a swarm, and might prove to be of importance in connection with the pathogenicity of the cultures and the production of immunity in the host, or they might represent nothing more than a mutation.

Here I may mention that in 1914 I advised that dysentery would probably occur in the Army, and suggested that the whole problem of dealing with this condition should be worked out in advance. Unfortunately the War Office did not accept the scheme of research which was submitted, so I had no opportunity of testing these points, which would have formed part of the general investigation.

Attempts, however, were being made at the Brown Institution to isolate the large forms of bacilli, when at the end of 1915 the work was interrupted, and I was asked by the War Office to organise and take out the Base Laboratory to Salonica. Soon after arriving I again advised that a systematic investigation should be carried out on dysentery with the object of obtaining an effective vaccine or serum against this condition, but once more I failed to obtain official approval, although I constantly gave expression to my views. As I had expected, the outbreak of dysentery, which at first was almost entirely bacillary in nature, arrived in due course, and it was not long before we were inundated with routine work. At the same time most of the staff became more or less ill and, while we were able to confirm the results already mentioned, it was impossible for me to carry the investigation much further.

The absence of official approval of my proposals no doubt was partly due to the fact that we came into strong conflict with the "Medical Advisory Committee" in Egypt, who concluded that the majority of cases of dysentery in the East were due to amœbic infection, and had criticised our diagnosis that the infection in Salonika was bacillary in nature. Lieut.-Col.

Buchanan, a member of the Committee, has since admitted in his presidential address before the Epidemiological Section of the Royal Society of Medicine, December 7th, 1917, that he knew "very little of dysentery on the practical side," and that the Committee had fallen into the error of mistaking body-cells in the discharge for parasitic amœbæ in case after case of dysentery; but this admission was made after we had returned from Salonika and it was therefore too late to undo the damage done by their first reports. However, the work was continued on my return, and that portion of the investigation on dysentery which is given in this paper would have been published in 1918 had I not included a criticism of the War Office and the Medical Advisory Committee for failing to take what I considered to be the necessary precautions against dysentery and for the erroneous diagnosis of cases in the East. As it was I was unable to get my paper published. Moreover, about the middle of 1918 I was ordered to rejoin the R.A.M.C., and it then became impossible for me to carry the matter further.

On returning from Salonika, as I have said, some additional experiments were carried out and some interesting results obtained. In the first place the long forms, after some difficulty, were isolated from ordinary "pure cultures" of various types of dysentery, from typhoid, and from certain coliform bacilli.

The medium used consisted of beef extract, 1 lb. to 2 litres of distilled water. To this, after filtration, were added 1 per cent. peptone, and ½ per cent. of sodium chloride. A solid medium was obtained in the usual manner by adding 2 per cent. of thoroughly washed agar. This was cleared with the yolk and white of egg, and to the clear agar were added 0·1 per cent. sodium bicarbonate, 0·5 per cent. of litmus solution and 0·5 per cent. maltose. The medium was now tubed and autoclaved.

The strains of bacilli to be tested were plated and replated several times to ensure purity. This was done by inoculating the condensation water of sloped tubes in a series of dilutions, and flooding this over the surface. Colonies on the last plates were then taken as pure cultures and placed on tubes of the medium described above. Observation demonstrated that these cultures contained a few units which were much larger and thicker than the normal bacilli, and that they were more numerous in the very young cultures than in older ones. Repeated subculturing from very young cultures caused a relatively large number of the big forms to grow, and after plating out such cultures on the surface of sloped tubes of the same medium, colonies containing a large number of the big forms were obtained. The cultures thus isolated agreed fairly closely with the original cultures in fermentation reactions and in agglutination tests, but the growths on solid media presented a much more spreading and wavy character. Some of the colonies when young appeared to be associated with a slowly acting dissolving material, similar to that described above, and subcultures from such colonies often failed to grow.

In cultures of the "large forms," the bacilli grew with considerable variation in length, thickness and shape, and a further series of experiments was made with a typical strain of the Shiga type of dysentery in order to separate if possible the different large forms, the same medium being used. A culture of the big forms which was isolated from this Shiga was grown under a nearly complete anaërobic condition and the growth was then plated out aërobically on sloped tubes of the maltose medium. Two distinct types of colonies were obtained. One, which will be referred to in future as type A, grew as large mottled colonies. Stained films and hanging drop preparations of this type showed many long, thin, wavy threads, sometimes, when observed under a $\frac{1}{12}$ oil immersion lens, extending half way across the field of the microscope. These threads are often twisted like a spirochæte (Fig 2). They vary considerably in length—some, possibly very young members, being quite short—whilst a few granules are sometimes present. The second type of colony, which will be called type B, grew at first as small grey colonies, but later spread considerably and became markedly mottled. Films and hanging drop preparations from this type present some very interesting morphological characters. The units, as a rule, are long like the first-mentioned thread forms, but thicker (Figs 3 and 4), and are often markedly swollen, either in the middle or at one end (Fig 4). The large swollen ends are sometimes split open, and are seen to have set free numerous granules; moreover a large number of these granules can be found free in all film preparations (Fig 3). How far these may be connected with the dissolving material already described has not yet been determined. Some units of a culture are granular and almost globular in shape, and these may attain the size of a yeast-cell or may be not much larger than a coccus.

On replating type B, a third form, or type C, was obtained. Colonies of this type are usually smaller, and are more waxy in appearance and less mottled. Film preparations show thick bacilli which grow considerably shorter than type B, but present the same swollen ends and the same granules as seen in that type (Figs. 5 and 6). The round granular forms are present in large numbers, especially in cultures that are several days old, where most of the units present a globular shape. In older cultures the protoplasm collects as a semilunar mass round the circumference of each unit; at least there is very little stain taken up by the rest of the cell (Fig. 7). In young cultures short, swollen, branched forms are often seen; these, as a rule, are roughly triangular in shape—that is, they present three short tapering outgrowths from a central granular protoplasmic mass. Longer branched forms are also to be seen (Fig. 6).

Some of the characters of the special forms A, B, and C are best observed when they are grown at between 25° C. and 30° C., but this is not essential. It may be noted also that although these forms were isolated on a maltose medium, subcultures made on to ordinary peptone-salt-broth agar show the characters equally well.

It is important to note that the large forms can always be isolated from the short or normal form, provided suitable means are employed, but it has not yet been ascertained for certain that the short forms can be recovered from any of the long or special forms, although cultures of these types may contain very short bacilli. Types A, B, and C show a tendency to grow these

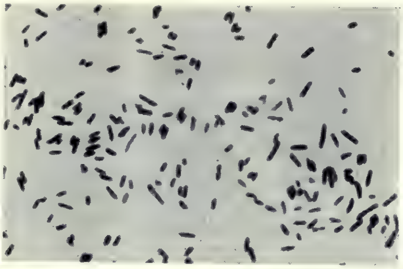

FIG. 1.—Normal type, 24 hrs. at 37° C.

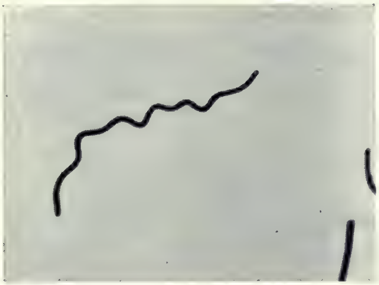

FIG. 2.—Type A, 5 hrs. at 37° C.

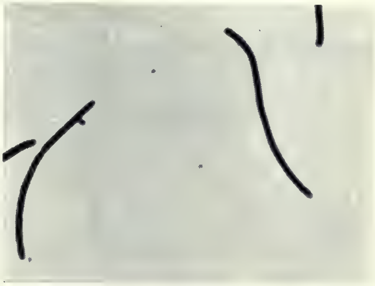

FIG. 3.—Type B, 5 hrs. at 37° C.

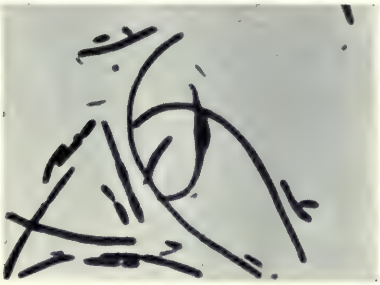

FIG. 4.—Type B, 24 hrs. at 25° C.

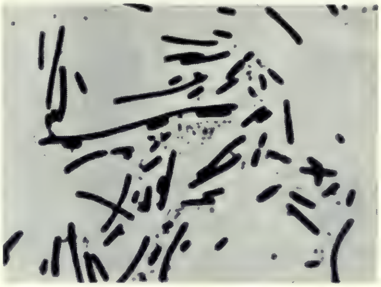

FIG. 5.—Type C, 5 hrs. at 37° C.

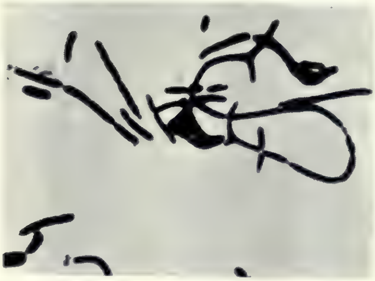

FIG. 6.—Type C, 24 hrs. at 25° C.

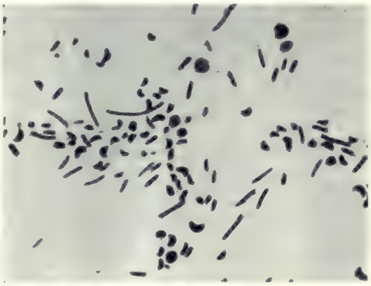

FIG. 7.—Type C, 3 days at 25° C.

CULTURES OF SHIGA'S DYSENTERY BACILLUS ON PEPTONE-SALT-BEEF-BROTH AGAR.

Twort. Researches on Dysentery.

forms mixed after a number of subcultures have been made, and this is particularly so with type A, which occasionally produces baccilli which, when isolated in plate cultures, show the characters of type B. Types B and C show greater variation in the shape of their units when first isolated from a normal culture than after repeated subculturing of the types.

In the case of certain of the coliform bacilli, the morphological differences between the different forms are much less marked than with the dysentery bacilli.

The exact relation of the "dissolving material" and the "granules" to the bacilli has not yet been determined, but it may be noted that they have never been grown apart from cultures of the bacilli.

Colonies of type C, and to a less extent those of type B, are often sticky and viscid like mucus, and this is usually more marked when they are grown between 25° C. and 30° C. In fermentation tests types A, B and C do not vary much from the standard small type, although on litmus-maltose-agar slopes type C produces marked acidity, type B fair acidity, while with type A there is often no acidity except near the condensation water. Types A, B and C agglutinate with dysentery agglutinating serum to a higher titre than does the normal type, but as spontaneous agglutination is very difficult to avoid with the special types, it is impossible to give detailed comparative tests. It is, however, quite clear from the experimental results that the special forms are not, as was at one time thought possible, distinct symbiotic bacilli, and the additional experiments carried out with typhoid and with coliform bacilli and their large forms confirm this view.

It may also be noted that the special forms do not appear to represent a type of degeneration, since they are best obtained by using good media and by keeping the cultures under good conditions.

It is, of course, possible that bacteria, like the spirogyra and other low forms of life, may not only multiply asexually, but also sexually. It may, in fact, be that a sexual form of multiplication is essential to the maintenance of a vigorous life after a certain number of asexual divisions, and it was thought that the special forms described above might represent the sexual units. Experiments carried out to test this were all negative. Mixtures of the special forms, after repeated subculture in broth, failed to give colonies of normal bacilli. If sexual forms do exist they must be present in the normal culture, since it is from this that the special forms are obtained. On the other hand, the fact that the special forms, when subcultured on ordinary agar, as they have been for several years now, do not reproduce normal cultures in rotation, is a strong argument against their being stages in a true life-cycle. It may be that they are nothing more than mutations, but if so it is a little strange that although a few can always be found in young cultures of dysentery bacilli, yet they never appear to survive under normal conditions when a normal culture is grown on ordinary laboratory media: the normal forms, although apparently producing the special forms, nevertheless soon outgrow them. It may be that the "dissolving material" plays a part in this process.

Another possible explanation appears to be that the large forms are specialised units which are produced by the normal bacilli for some special purpose. They may assist in producing assimilable food or protective substances for the normal bacilli whose chief function is to reproduce. If such is the case it might explain why the special forms do not normally survive in cultures made on laboratory media. It might also explain certain points regarding successful infection, incubation period, the termination of infection, and the production of toxins, antitoxins, etc. In this connection it is interesting to note that in specimens from the human body of such material as fæces and urine, long forms of bacilli are often present in considerable number, and this is especially so in specimens from the upper part of the intestinal tract in cases of infantile diarrhœa and vomiting, and in specimens of urine in cases of cystitis; and it does not seem at all impossible that these forms may play an important *rôle* in producing the toxic effects in such conditions. This is a point that should be investigated further, for it might lead to the production of more efficient vaccines and possibly also of antitoxins.

Some experiments were carried out to test the toxicity of the special forms for animals. In most cases mice and rabbits were used, and the results appeared to show a greater toxicity of these forms than the normal cultures, but they were not sufficiently marked to draw any definite conclusions. It is, however, worthy of note that the special forms appear to lose their toxicity rapidly when they are isolated from the small forms, and it may be that their power of producing toxic substances is marked only in those units which are produced directly from the small forms. The fact that in a normal culture the special forms appear to be produced in this manner and then die out without showing any evidence of multiplication by simple division, gives support to this suggestion. If effective toxin-producing units must be produced directly from the small forms, it is possible that the use of the dissolving material is to destroy them after they are no longer of any use to the bacterial community, or the dissolving up may be a means of setting free the enclosed toxic substances. In the case of the micrococcus which was isolated from vaccinia, the breaking down of the organisms may extend to the whole culture, and in a few hours no living units may be left; but conditions of cultivation outside the body are very artificial, and it seems improbable that in the infected body this process would extend further than was beneficial to the life of the bacterial community.

If then, as seems possible, the function of some of the special forms of dysentery bacilli is to produce toxins or other substances for the protection of the bacterial community, then any conditions which might lead to the production of these forms would assist the normal bacilli to survive and multiply when they gained entrance to the human body. In this connection my wife, then Miss Banister, devised and carried out a series of experiments to test for the production of large forms in media that contained a high percentage of sodium chloride, the idea being that the climatic conditions which favour an outbreak of dysentery would also produce a higher concentration of salt in their environment. In these experiments the maltose medium described above was used as a basis, and 1 per cent. of sodium chloride was added in place of 0·5 per cent. A pure growth of normal Shiga bacillus was inoculated on this medium, and repeated subcultures made on to fresh tubes. In these cultures large forms were produced as in the previous experiments, but in addition a few extremely large branching threads were observed. Their length was considerably more than the diameter of the microscopic field, and they appeared more like the mycelium of a mould than anything else. These threads contained granular protoplasm, and as a rule remained but faintly stained. The experiments were repeated several times with the same results, but all attempts to isolate these forms failed so that it was impossible at the time to investigate them further, and their significance could not therefore be determined. It may be noted, however, that great care was taken to eliminate contaminations, and I think this possibility can be excluded.

More recently, with the assistance of my wife, experiments have been carried out for the Medical Research Council on influenza, and somewhat similar results have been obtained. We hope to publish a paper on these investigations shortly.

SUMMARY.

Three types of special forms can be isolated from dysentery and allied bacilli. Type A shows moderately thick but long bacilli, which may twist like a spirochæte. Type B shows long thick rods; some swollen in the middle or at one end, and these may break open and set free granules. The bacilli are associated with round forms and many free granules. Type C bacilli are like type B, but shorter. There are many granules and round forms, also thick branching units. In older cultures most of the units are globular, and the protoplasm may collect as a semi-lunar mass round the circumference. A filter-passing dissolving material may be present in cultures of dysentery bacilli, etc., which breaks down the bacilli into granules. The special types A, B and C are produced by normal bacilli, but some smaller units are always present. The special forms are probably not stages in a true life-cycle, as the normal type is not produced in rotation, and it is doubtful if they are mutations. Special types may be produced for performing special work, and types in "pure culture" may be likened to bees in a swarm. They are possibly of importance in connection with incubation period, successful infection, and production of immunity. Sexual multiplication is not proved, but may exist in normal cultures.

THE RESPIRATORY EXCHANGE OF SURVIVING MOUSE TISSUES, NORMAL AND NEOPLASTIC.

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IN a previous number of this Journal one of us in collaboration with W. E. Gye (1) published an account of experiments bearing upon the oxygen consumption of normal and cancerous mouse tissues *in vitro*. The reasons for carrying out the experiments *in vitro* instead of by the preferable method of *in vivo* were detailed there and need not be entered into again. Shortly stated, the oxygen consumption was ascertained in those experiments by measuring the amount of oxygen which disappeared from a known amount of defibrinated rabbit's blood, after incubation with normal or cancerous mouse tissues. We also attempted to estimate the carbon dioxide of the blood, but the measure of success which attended the estimation of oxygen was not repeated in the case of the other gas, for the figures obtained were so erratic as to be quite unusable. It was impossible to strike any average which would give a reasonable respiratory quotient, and with it information as to the probable food substances mainly consumed.

It may be recalled that the respiratory quotient expresses the ratio of the carbon dioxide excreted to the oxygen absorbed. In the case of carbohydrates these two volumes are equal and the quotient is unity. When fats are metabolised there is relatively less carbon dioxide given off, and a quotient of 0·71 is found. The quotient for proteins varies slightly, but is usually about 0·81. Sometimes a respiratory quotient exceeding unity is observed, as in the case of some animals after a meal rich in carbohydrate. This has usually been interpreted as meaning a conversion of part of the carbohydrate into fat. A quotient below 0·7 generally means incomplete oxidation with the formation of intermediate metabolic products. If the respiratory quotient be known an idea is obtained of the respective food-stuffs being burnt up, and for this reason it seemed desirable to ascertain the quotient for a number of different tissues and tumours.

In the present communication an account will be given of the respiratory exchange as ascertained by a different method, which, although in some ways inferior to the previous method, has nevertheless supplied us with results which in their consistency have surpassed what we had reason to expect.

The method used was to measure the gaseous exchange of a tissue emulsion exposed at blood heat in a gas chamber. The difficulty of providing a suitable apparatus was overcome by using an ordinary Barcroft apparatus as made for the analysis of gases in 1 c.c. of blood. Krogh (²) has pointed out that this apparatus can be used for respiratory estimations on small animals, and has devised a modified form which is an extremely sensitive respirometer, and can measure the oxygen absorption of a single insect egg weighing 2 mgrm. For mouse tissues with a high oxygen consumption, such as kidney, the ordinary Barcroft apparatus gives quite good results, but for most tumour tissues which have a lower gaseous interchange we have found it necessary to have an apparatus made* which was more sensitive, and which at the same time allowed more tissue to be introduced. The apparatus has been calibrated for the varying conditions during the experiment by the method described by Hoffmann (³).

When an experiment is to be carried out, a large water-bath provided with an adequate mechanically driven stirrer is heated up to 38° and kept steadily at this temperature. The respirometer is then hung on the bath with two bulbs completely immersed. This preliminary warming shortens the period necessary to attain equilibrium later. The animal is killed, and the tissue to be used is rapidly excised and minced with curved scissors until an apparently homogeneous emulsion is obtained. Part of the emulsion is spread evenly on a rectangular slip of ground-glass, the amount being ascertained from the difference in weight of glass before and after. Weighing has been done on a Collot balance, which reduces the time taken and evaporation to a minimum. The lower portion of the slip of glass must be kept free of tissue, otherwise there will be contact with the alkali which is added later. The glass slip with the tissue is placed in one bulb of the respirometer, with a corresponding glass slip in the other, and both bulbs are filled with expired air and replaced on the apparatus, which is returned to the water-bath. The time elapsing between the death of the animal and the commencement of incubation has varied between ten and fifteen minutes.

Expired air has been chosen because it is relatively constant in its composition, is saturated with water vapour, and contains carbonic acid at a tension approximating to that usually experienced by the tissue. Human expired air has of course been used, but it is probable that mouse expired air does not vary very widely from it in its composition. It was hoped that in using expired air, equilibrium, especially of carbonic acid exchange, would be sooner established, and the results seem to harmonise with the view.

After ten minutes, during which the apparatus is warming up, the taps are turned off and the position of the manometer menisci recorded. Observations are made usually at ten-minute intervals for forty minutes, the figure for the first ten minutes being rejected, as it is usually about fifteen minutes after incubation begins before a steady rate of gaseous exchange is established. Any absorption—that is, a contraction of volume on the side of the apparatus containing the tissue—observed during these forty minutes will represent a surplus of oxygen consumed over the carbonic acid produced, whilst an expansion will represent a surplus of carbonic acid. If no movement be noted it is obvious that the oxygen and carbon dioxide exactly balance, as they do in the case of a pure carbohydrate metabolism with a respiratory quotient of unity. To ascertain the total amount of oxygen being consumed, 1 c.c. of warm 6 per cent. KOH is added to each bulb to absorb the carbon dioxide, and after a suitable interval of ten or fifteen minutes the excursion of the manometer is again read at ten-minute intervals. If the gaseous exchange is constant over the two periods observed a respiratory quotient can be established. The disposition of the apparatus did not allow the maintenance of an atmosphere of uniform composition in the two periods, but as the conditions were maintained in the same way in successive experiments comparative results could be obtained. The duration of the period of observation has been largely determined by the circumstance that we are dealing with cells which are beginning to die, and that the result must be obtained before they have gone too far on their way. In general the displacement of the manometer has been fairly uniform over successive periods of ten minutes, and the following protocol is taken as an example of what may be expected to occur.

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* The apparatus has been made for us by Messrs. Orme & Co., 148, High Holborn, W.C. 1.

1/7/20.—Mouse bearing a transplanted tumour of carcinoma 155 was killed at 2.5 p.m.; the tumour was rapidly excised, and after being freed from all apparent necrotic areas was emulsified with curved scissors. Of this emulsion 286 mgrm. were spread on a glass slip over a surface of 800 square mm., placed in the respiratory chamber, and the respirometer returned to the water-bath at 2.15 p.m. At 2.24 p.m. both chambers were shut off from the air, and the recording of the manometer changes commenced.

Absorption.

p.m.	mm.	p.m.	mm.
2.24	0	3.12	2
2.30	1	3.22	9·75
2.40	3·75	3.32	17·5
2.50	6	3.42	24·5
3	8		

After addition of 1 c.c. 6 per cent. KOH to each bulb, the changes recorded in the second column were obtained: the 7 mm. absorption occurring between 2.30 and 3 equalled 13·33 c.mm. at 0° C. and 760 mm. Hg.; the 22·5 mm. absorption between 3.12 and 3.42 equalled 41·39 c.mm.

$$\frac{41{\cdot}39 - 13{\cdot}33}{41{\cdot}39} = \frac{CO_2}{O_2} = \text{respiratory quotient } 0{\cdot}678.$$

The amount of oxygen consumed was equivalent to 4·8 c.c. per kilogramme minute.

The value of the respiratory quotient thus established will also depend on the evenness of the respiratory exchange during the two periods. This has been controlled in a separate apparatus, where part of the tumour emulsion was incubated over alkali from the start, and the following absorption noted at ten-minute intervals. Starting at zero the successive readings were: 2·25, 5·5, 8, 10·25, 12·25, 14·5, 16·75, 19, 21. The successive increments are sufficiently close to warrant the assumption that there is no appreciable alteration in the character of the respiratory exchange during the period of observation.

The first mouse-tissue to be tested was minced kidney, as this has a very active metabolism, and the results of six observations on the kidneys of different mice are summarised in Table I.

TABLE I.—*Kidney.*

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·893	20·0	61	230
0·884	18·2	93	,,
0·875	12·7	124	,,
0·880	19·2	85	,,
0·884	22·4	76	,,
0·883	15·0	102	,,

Average respiratory quotient 0·883, ± 0·002.

The mean error of the series does not affect the second decimal place, and the respiratory quotient of surviving mouse kidney may be taken as having a mean value of 0·88. The oxygen consumption per kilogramme minute has fluctuated rather widely, but the higher values obtained agree with the average, 21·3 c.c., found for kidney suspended in rabbit blood. In spreading an emulsion of kidney substance on a glass slide it is not always possible to maintain exact uniformity, and the actual quantity used in covering a given surface has varied considerably, as will be seen from the figures in column 3. Comparison of column 2 with column 3 shows an inverse proportionality: the more tissue used above a given optimum the lower the rate of oxygen consumption. As different tissues vary in the facility with which they can be minced and spread, the method is not a good one for measuring comparative oxygen consumptions, although it gives comparative respiratory quotients. The figures for six experiments on liver-tissue are given in Table II (page 248).

The mean error of the series is here ten times as great as that with kidney, and the quotient is appreciably lower. Liver-tissue has been found to be much more labile than kidney, for where the latter will consume oxygen at an undiminished rate for two hours at least, the liver-tissue will show a falling off much sooner. For this reason we suspect that the respiratory quotient struck for liver is a little too low.

TABLE II.—*Mouse Liver.*

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·868	7·6	82	230
0·826	5·8	80	,,
0·736	7·0	126	460
0·759	7·8	156	,,
0·804	12·5	119	,,
0·887	11·1	154	,,

Average respiratory quotient 0·813, ± 0·024.

In some cases, but not in all, a portion of the liver emulsion was fixed and stained for glycogen. The left-hand portion of Plate-fig. 1, shows the abundance of fine granules of glycogen before incubation, whilst the right-hand portion shows the diminution after one and a half hours' incubation. The renal parenchyma of the mouse does not usually show any glycogen, although its higher respiratory quotient means a more extensive use of carbohydrate during the survival period tested.

Because most mouse tumours are, after all, progressively growing mammary epithelium, we give in Table III figures for the mammæ of five pregnant mice, killed during the last week of gestation. Histological examination of part of the organ showed in all cases a considerable degree of epithelial growth with reduction of the fatty embedding tissue.

TABLE III.—*Mamma.*

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.	Date of pregnancy.
1·000	12·2	136	480	16th day.
0·935	6·7	106	,,	?
0·920	8·5	212	800	18th day.
0·853	10·0	129	480	19th day.
0·861	9·4	158	800	20th day.

Average quotient 0·914, ± 0·027.

This is the highest quotient yet found for normal tissues, and if the individual figures are examined, it will be noted that there is a tendency for it to fall as the end of gestation is approached.

The submaxillary salivary gland, a large readily excisable organ in the mouse, was also examined, and the figures are given in Table IV.

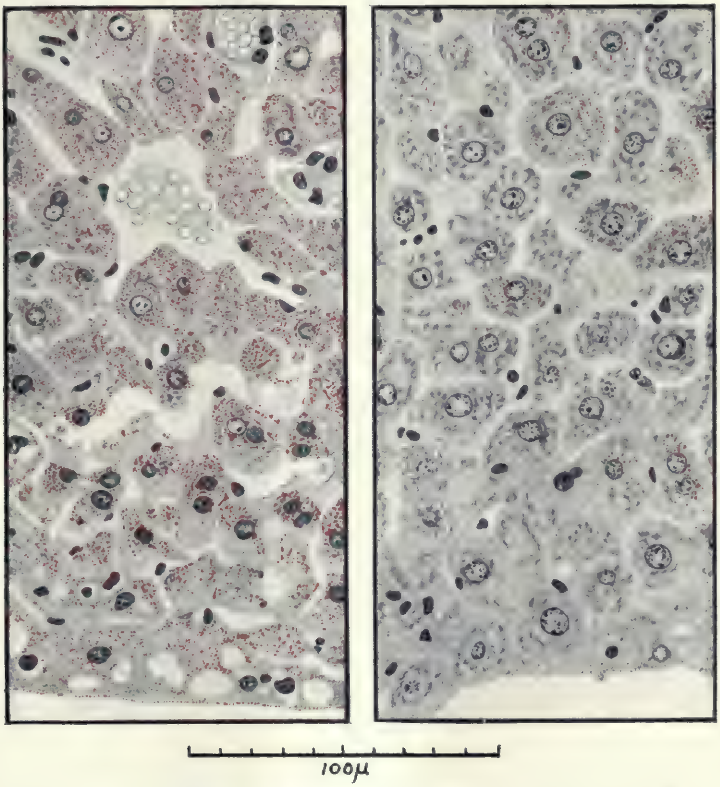


FIG. 1.—Liver stained for glycogen. Left hand: before incubation. Right hand: after 2½ hours' incubation. × 410.

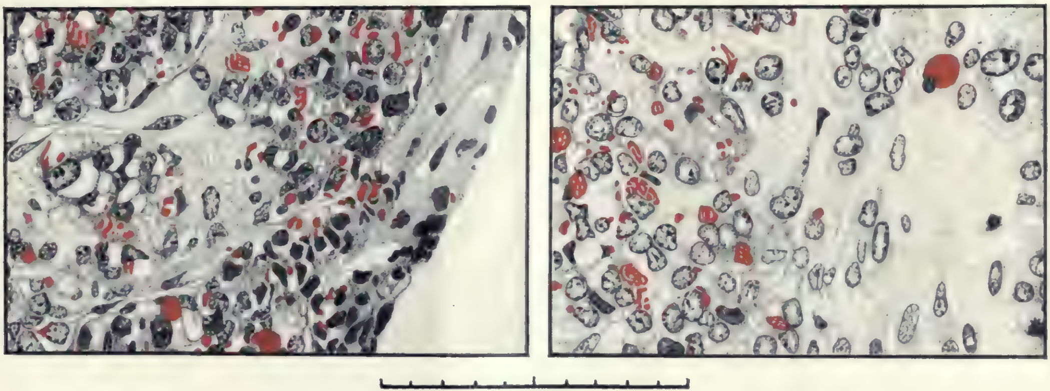


FIG. 2.—Carcinoma 113 stained for glycogen. Left hand: before incubation. Right hand: after 1½ hours' incubation. × 410.

Russell and Woglom. Respiratory Exchange of Mouse Tissues.

TABLE IV.—*Submaxillary Salivary Gland.*

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·901	9·3	70	240
0·739	10·8	112	480
0·845	7·0	134	,,
0·829	10·5	134	,,
0·832	12·9	201	800
0·901	8·3	174	600

Average respiratory quotient 0·841, ± 0·024.

Before discussing the results obtained with these four tissues, the findings on six transplanted mouse-tumours will be given. Five of these were mammary carcinomata, and one, 37 S., a spindle-celled sarcoma. The carcinomata present great differences in their speed of growth, the fastest being tumour 206, followed by 63 and 113, whilst the two strains 72 and 155 grow very slowly. The two latter strains present a more adenomatous type of growth than the three others, which are predominantly alveolar in structure. As previously shown (1), the rate of oxygen consumption by these tumours falls with diminishing speed of growth, exception being made for the slowly growing adeno-carcinomata where there is a rise above what might be expected.

In assessing the rate of growth of different tumour strains, the outlines of the tumours produced at intervals of one or two weeks have been compared, but this is at best only a rough comparison. The actual increment is the resultant of two opposing factors—the multiplication of cells on the one hand, and the death of many cells on the other. The visible speed of growth need not therefore represent the actual rate of multiplication of the tumour-cells, for the rate of necrosis may vary considerably, and obscure the varying production of new cells by different parenchymata. Only pronounced differences in the visible rate of growth of different tumour strains can be regarded as significant.

TABLE V.—*Sarcoma* 37 *S.*

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·936	7·6	201	460
0·942	7·0	180	,,
0·901	5·9	395	800
0·948	7·0	201	460
0·976	5·4	188	,,
0·946	12·0	150	,,

Average respiratory quotient 0·941, ± 0·010.

37 S. is a very rapidly growing spindle-celled sarcoma which arose during

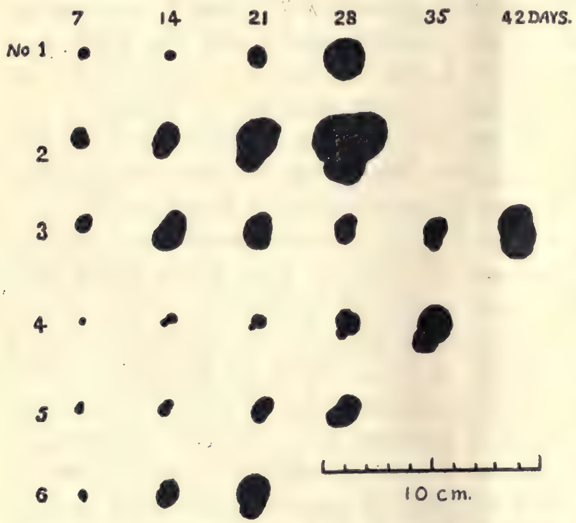
the transplantation of a mammary adeno-carcinoma in the manner described by Haaland (4). On inoculation it grows in a high percentage of animals, but many of the tumours later recede and disappear.

TABLE VI.—*Carcinoma* 206.

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·916	5·3	144	460
1·000	8·2	153	,,
0·795	5·9	302	800
0·933	3·5	202	460
0·810	5·2	255	800
0·753	4·2	306	,,

Average respiratory quotient 0·868, ± 0·039.

Carcinoma 206 is an alveolar carcinoma of the mamma. The transplanted growths are extremely prone to undergo spontaneous absorption, and induce a highly refractory state to subsequent inoculation of the mice (5). The rate of growth of individual tumours varies extremely in this strain; sometimes it will grow even faster than 37 S., but at others it will slow up very much. On Text-fig. 1 are given the outlines at weekly intervals of the six tumours used in the respiration experiments in the same order. From this it will be seen that there is a tendency for the respiratory quotient to rise with increasing speed of growth.



TEXT-FIG. 1.—Chart showing rate of growth of the six tumours of carcinoma 206 tested.

TABLE VII.—*Carcinoma* 63.

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·853	7·9	154	460
0·794	8·0	145	,,
0·841	5·4	186	400
0·854	7·2	164	460
0·771	6·2	259	800
0·798	6·4	280	,,

Average respiratory quotient 0·818, ± 0·014.

Carcinoma 63 is predominantly alveolar in structure. It grows more slowly than the two previous tumour strains, but is much steadier in its behaviour.

TABLE VIII.—*Carcinoma* 113.

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·924	4·7	296	800
0·980	4·6	298	,,
0·948	2·9	211	,,
0·901	4·2	204	,,
1·000	4·3	327	,,

Average respiratory quotient 0·951, ± 0·018.

This tumour is also an alveolar carcinoma and grows much more slowly than carcinoma 63. Its special feature, however, is the constant presence of considerable quantities of glycogen as shown in Plate-fig. 2. The left-hand portion represents a fragment of the emulsion before incubation, and the right-hand a similar portion after one and a half hours' incubation. In this time no appreciable diminution could be detected by the microscope in the amount of glycogen present. This is scarcely what might have been expected from the very high respiratory quotient given by all the five samples. If the microscopic behaviour of the glycogen in the liver and this tumour be compared with the respective quotients found there is a pronounced discordance. The extremely fine character of the glycogen granules in the liver-cells will of course facilitate the process of glycogenolysis, whether the carbohydrate be needed for immediate combustion or not.

TABLE IX.—*Carcinoma* 72.

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·629	4·1	221	800
0·837	4·0	317	,,
0·649	3·2	310	,,
0·714	4·7	283	,,
0·732	4·3	307	,,
0·752	3·9	278	,,

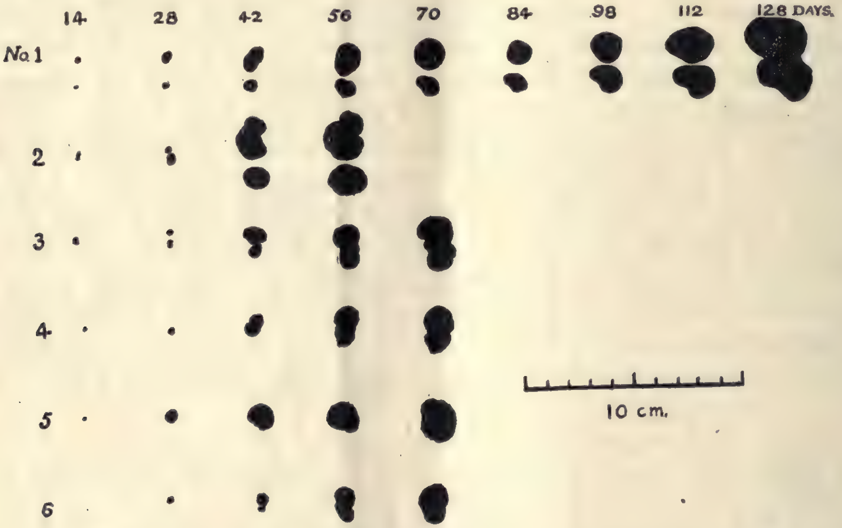
Average respiratory quotient 0·719, ± 0·031.

TABLE X.—*Carcinoma* 155.

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·683	6·5	330	800
0·661	4·8	351	,,
0·706	5·3	143	460
0·697	4·0	253	800
0·617	4·7	306	,,
0·678	4·8	286	,,

Average respiratory quotient 0·674, ± 0·013.

Carcinoma 72 and carcinoma 155 are very similar in structure and type of growth. The general type of structure is predominantly acinous, the acini being admittedly imperfectly constructed, with the lumen often drawn out into a long slit. Both grow extremely slowly, carcinoma 155 being even slower than 72. Text-fig. 2 shows at fortnightly intervals the silhouettes of the six tumours of 72 which were tested, and it will be seen that the unusually rapid growth of the second specimen gave a much higher respiratory quotient than the other five. The abnormally low quotients given by 155 are possibly due to imperfection in the method used, although they may be produced by an incomplete oxidation of the intermediate products of katabolism.



TEXT-FIG. 2.—Chart showing rate of growth of the six tumours of adeno-carcinoma 72 tested.

An epithelioma which started on the back of a male mouse and which is being successfully transplanted, gave on the three occasions tested respiratory quotients of 0·846, 0·755 and 0·862. In rate of growth this tumour equals carcinoma 113, but its excessive tendency to undergo spontaneous absorption and to keratinise renders the securing of suitable material difficult. Only one spontaneous tumour has been examined and the quotient 0·785 found. The tumour was a hæmorrhagic adeno-carcinoma of the breast, and the material tested came from a fairly rapidly growing mass which recurred after operation.

The above average respiratory quotients show greater differences in the case of the tumour tissues than in the case of the four normal parenchymata tested, and the differences in the case of the tumours present a hitherto unsuspected feature. The consistency of the results obtained with the individual tissues inspires a certain degree of confidence, but as they are the first attempt to fix a respiratory quotient for different mouse tissues, and as the method of analysis is admittedly defective, caution in interpreting the results is indicated. Although much experimental work has been done on the oxygen consumption of organs and tissues, there are few cases where a consistent respiratory quotient has been established for surviving tissues.

Vernon (6) obtained a quotient of 0·85 for the rabbit kidney perfused with oxygenated Ringer at room temperature. This is fairly close to the figure found by us for mouse kidney.

Cohnheim and Pletnew (7) found for the cat's intestine a quotient from 1·07 up to 1·29, but their method involved the destruction of the lining epithelium and bacteria by a 0·17 per cent. solution of corrosive sublimate. In a large series of experiments on the heart-lung preparation from dogs, Starling and Evans (8) obtained a quotient of 0·85 for the normal dog. The hind limbs of a dog were found by von Frey and Gruber (9) to give a quotient slightly above unity. Battelli and Stern (17), who worked with minced tissues of the dog, found for the heart and liver quotients of 0·72 and 0·58 respectively. Harden and Maclean (10) obtained quotients varying from 0·9 to 1·3 with minced rabbit muscle. Rabbit liver was investigated by Fedeli (18), and a quotient of 0·725 ascertained.

In addition to the four normal tissues a few experiments were carried out by us on embryonic tissues, which share with tumours a rapid proliferation rate. Three were done on emulsions of the total embryo, and gave quotients varying from 1·3 to 1·1, but the rate at which the excess of carbon dioxide came off dropped rapidly during the first thirty or forty minutes of the incubation. The abnormality of the quotient with this tissue is probably due to the lactic acid formation in the muscle cells driving out carbon dioxide from pre-existent combination. Fletcher (11) has shown clearly, both for minced rabbit and rat muscle, that there is a rapid rise in the amount of lactic acid present during the first half hour, and an excessive evolution of carbon dioxide in the same period. On two occasions the respiratory exchange of an emulsion made from mouse embryo skin was tested, and on each occasion a quotient of unity was found. The experiments on embryonic tissues have been hampered by an unusual run of sterility in our breeding stock, and will have to be pursued as occasion offers.

Whilst many observations have been made upon the respiratory exchange of the embryos of cold-blooded animals and upon the developing eggs of hens, but few attempts have been made with warm-blooded animals. Cohnstein and Zuntz (12) made the attempt on sheep embryos, but did not succeed in establishing a respiratory quotient. Bohr (13) had more success with the guinea-pig, and concluded that the total gas exchange was about the same as for the mother with a respiratory exchange about unity. His figures, however, show wide variations in the different experiments. That a developing mammal should have a respiratory quotient about unity stands in marked contrast with the low quotient of 0·71 found in the developing chick (14), for the former means pure carbohydrate metabolism and the latter a pure fat metabolism.

It was already noted that the quotient of the mamma of pregnant mice was apparently higher about the sixteenth day than at later stages of gestation. This fall might be correlated with the establishment of lactation, the metabolic exchange of the gland passing from that of pure development to one of functional activity. Before the onset of the latter the gland would appear to be burning carbohydrate only. Kidney, liver and salivary gland all have quotients pointing to a mixed metabolism, but in default of any estimate of the probable nitrogenous metabolism of the mouse, it scarcely seems feasible to arrive at approximate figures for the respective amounts of food-substances which would give the various quotients. In any case the imperfections of the method of arriving at the respiratory quotient do not justify us in drawing any but the broadest and most apparent conclusions.

It may be here recalled that Cramer (15), in comparing the gaseous exchange of rats bearing inoculated tumours with normal rats, found no essential difference in the respiratory quotient. Chisolm (16) came to a similar result in the case of mice. The relatively small part which a necrosing tumour occupies in the total gaseous metabolism of the animal might readily obscure such differences as we have found in the observations detailed above.

In general it may be stated that when one tumour strain is compared with another there is, with increasing rate of growth, a rise in the respiratory quotient. The glycogen-rich tumour 113 makes an important exception to this statement, for although its rate of growth is slow, yet it gives the highest average quotient found for neoplastic tissues. Its large store of intracellular glycogen gives it a superabundant supply of carbohydrate to draw upon, although there is scarcely any histological evidence of reduction in the amount of glycogen after one and a half hours' incubation. For the greater part of this period, however, the tumour may, like the kidney, be drawing upon invisible carbohydrate. The storing of glycogen by this tumour parenchyma may be looked upon as a pathological excess over its actual needs.

Microscopic examination of the different tumour emulsions has shown that all contain plenty of fatty material, but it is only the very slowly-growing tumours 72 and 155 which give a quotient representing the exclusive combustion of fats. An interesting confirmation of the fat metabolism prevailing in these two strains was obtained by comparing the survival of the mitochondria. Whereas the mitochondria of the other tumours and normal tissues were well preserved after one and a half hours' incubation, in these two strains they underwent a rapid degeneration and disappeared. The histological evidence of this change will be dealt with in a later communication by one of us (B.R.G.R.).

In conclusion, attention may be drawn to the circumstances that these experiments do not deal with the respective part played by different food-substances participating in the building-up of a given tumour parenchyma, but only bear upon the sources from which the tumour derives the necessary energy. The distribution of this energy between energy of growth and energy of function is at present impossible in view of the elusive character of function in tumours. In so far as a conclusion may be drawn, the experiments do not go further than showing that rapidly-growing tumours draw more on carbohydrates for their energy, whilst slowly-growing more differentiated tumours apply themselves to fats.

SUMMARY.

Respiratory quotients were obtained for surviving normal and tumour tissues of the mouse by suspending emulsions in alveolar air in the Barcroft blood-gas apparatus. The results agreed very closely in successive experiments with each normal tissue. Greater variability was found with tumours:

Normal.			Tumour.	
Kidney	0·883±0·002	Rate of growth ——→ ← Differentiation.	37 S.	0·941 ± 0·010
Liver	0·813±0·024		206	0·868 ± 0·039
Mamma	0·914±0·027		63	0·818 ± 0·014
Submaxillary gland	0·841±0·024		113	0·951 ± 0·018
(Total embryo	1·3—1·1)		72	0·719 ± 0·031
(Mouse embryo skin	1·0)		155	0·674 ± 0·013

The tentative conclusion is drawn that the more rapidly-growing, undifferentiated tumours draw their energy from carbohydrate, the more slowly-growing from fats.

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ERRATUM.

In Dr. Cramer's paper, "On Glandular Adipose Tissue," page 190, line 19, "*on* material" should read "*no* material."

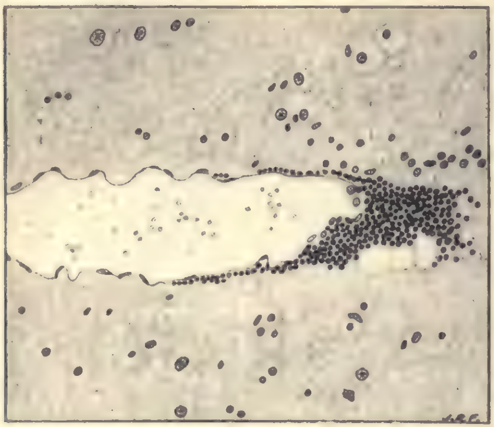

Fig. 1.
Mac. cynomolgus. Spontaneous infection. Haem. and eosin. Obj. $\frac{1}{8}$". Oc. 4 Zeiss.

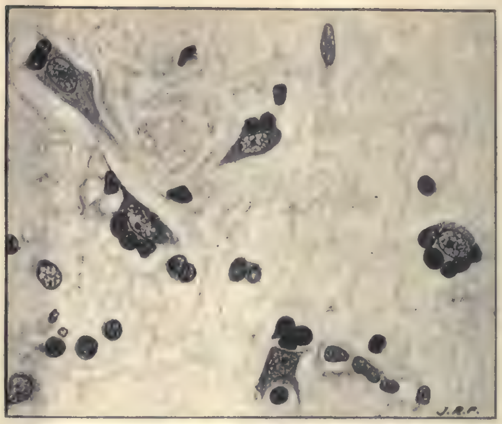

Fig. 2.
Mac. cynomolgus. Passage I. Sacral cord. Haem. and eosin. Obj. $\frac{1}{8}$". Oc. 4 Zeiss.

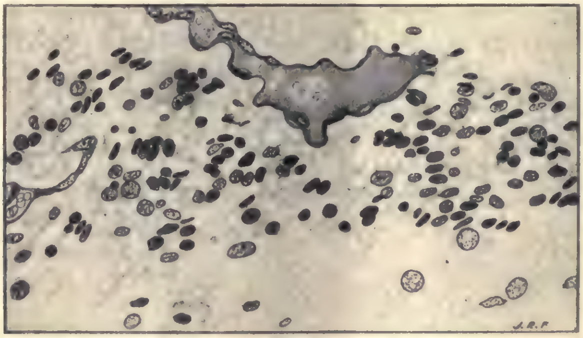

Fig. 3.
Mac. cynomolgus. Passage I. Rt. basal ganglia. Haem. and eosin. Obj. $\frac{1}{8}$" Oc. 4 Zeiss.

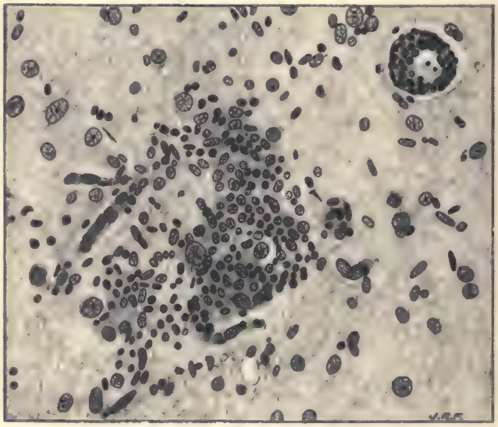

Fig. 4.
Rabbit. Passage I. Frontal lobe. Haem. and eosin. Obj. $\frac{1}{8}$". Oc. 4 Zeiss.

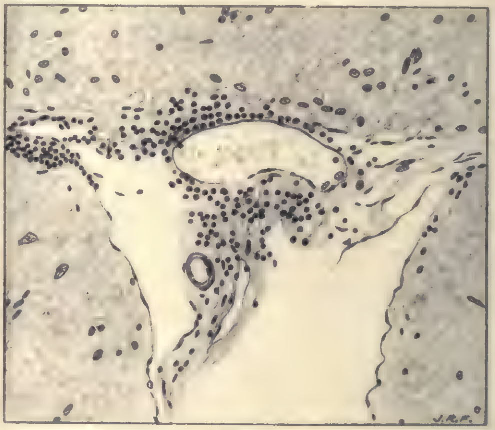

Fig. 5.
Rabbit. Passage I. Mid-brain. Haem. and eosin. Obj. $\frac{1}{8}$". Oc. 4 Zeiss.

TRANSMISSION OF EXPERIMENTAL *ENCEPHALITIS LETHARGICA* IN SERIES IN MONKEYS AND RABBITS, WITH NOTES ON A SPONTANEOUS INFECTION IN A MONKEY.*

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In a previous number of this Journal (1) an account was given of the successful transmission of *encephalitis lethargica* to a Patas monkey. The present article deals with the transmission of the disease in series—a detail necessary to complete the proof that the disease is caused by a living virus. At the same time an account is given of a spontaneous infection in another monkey.

PASSAGE I.

The virus was obtained from the successfully inoculated Patas monkey. Small pieces of the cervical cord, pons, basal ganglia and cortex were crushed in a conical glass vessel with a glass rod and then emulsified in 20 c.c. of sterile normal saline. The supernatant fluid was then drawn into a syringe and inoculated into two *Macacus cynomolgus* monkeys. Each animal received 1 c.c. of the emulsion intracerebrally and 5 c.c. subcutaneously.

Symptoms and Lesions of the Experimental Animals.

A. *Macacus cynomolgus* No. 1. Large male.

28/11/19.—Inoculated.
14/1/20.—No abnormal symptoms have been noticed.
10/2/20.—Animal quite fit.
12/2/20.—Reinoculated with the same virus.
12/3/20.—No abnormal symptoms have appeared to date.
14/5/20.—Ditto.

B. *Macacus cynomolgus* No. 2. Female.

28/11/19.—Inoculated.
14/1/20.—Quite healthy; no abnormal signs so far.
12/2/20.—Reinoculated.
16/2/20.—Quite fit.

* The expenses of this research were defrayed by the Medical Research Council.

23/2/20.—Has a cold—running at the nose, etc.

11/3/20.—Does not appear to be quite well; some trembling of arms and tendency to shake and rotate its head.

12/3/20.—Condition similar. This monkey, which was previously very playful, now refuses to play. Stumbles when walking on the floor, and its whole gait is clumsy.

13/3/20.—More lively; seems rather better and eats well.

15/3/20.—Quieter again to-day; will not play. There is a slight conjunctivitis of right eye, and the animal is very thirsty.

17/3/20.—Condition similar.

22/3/20.—Seems worse to-day; still has slight cold in the head. The animal might be described as being slightly somnolent; keeps eyes shut most of the time. When roused is querulous. Appears to have difficulty in swallowing and stretches out its neck while drinking.

23/3/20.—Seems slightly better.

27/3/20.—Condition worse again; still difficulty in swallowing.

31/3/20.—Hind legs are now weak and it can no longer stand erect as it used to. Twitches its head.

1/4/20.—Condition similar.

11/4/20.—This morning the animal was noticed to be very excited and jumping about in its cage. Soon after this it cried out and had a severe epileptiform fit, which lasted about an hour. It lay on its back moving its arms and legs in a purposeless manner. The eyes were open and the tongue extended and directed a little to the right. Later on the limbs became rigid and tremulous. The condition then gradually passed off, although the animal stumbled about for some time after. By the afternoon the animal had recovered considerably, but it was very weak and could not climb back into the cage.

12/4/20.—Condition improved. Could climb back into cage; still difficulty in drinking.

14/4/20.—Condition as above.

19/4/20.—Much weaker and stumbles a lot.

21/4/20.—Weaker. Killed by coal-gas.

Autopsy.—Apart from a distinct dryness and almost complete absence of fat in the subcutaneous tissues, nothing abnormal was observed in any of the internal organs. Macroscopically sections of the brain showed congestion of the grey matter. Pieces of the brain were placed in 33 per cent. glycerine for preserving the virus, while other portions were fixed in 10 per cent. formalin-saline for histological purposes.

Histological Examination of the Brain of Macacus cynomolgus *No.* 2.

Left frontal lobe.—Meninges in places show a slight increase of cellular elements, chiefly lymphocytes. The vessels of the cerebral substance are congested and often contain hyaline thrombi. The pyramidal cells appear normal.

Left parietal lobe.—In the cerebral substance there are a few small areas of perivascular infiltration, consisting chiefly of glial cells, lymphocytes, and a larger endothelial type of cell.

Left occipital lobe.—In one area there is a distinct perivascular small-celled infiltration and numerous hyaline thrombi.

Right basal ganglia—anterior.—Ganglion cells well formed; Nissl granules prominent.

Right basal ganglia—lenticular nucleus.—In this region the ganglion cells are frequently degenerate and shrunken; the nuclei often eccentric and staining deeply.

Right basal ganglia—posterior.—Condition as above; vessels frequently contain hyaline thrombi.

Section through caudate nucleus—left.—Vessels dilated, numerous hyaline thrombi; multiple hæmorrhages into the perivascular sheaths and surrounding tissues. In places distinct glial proliferation. Ganglion cells degenerate and often surrounded by glial cells.

Section through upper portion of corpora quadrigemina.—Many degenerate ganglion cells in lower part; the degenerate cells are to be found alongside healthy cells which have well-marked Nissl granules. There are numerous hæmorrhages to be seen in this section.

Section through pons at level of fifth root.—In many of the ganglion cells the Nissl granules are distinct, but among them are to be seen what appear to be degenerate cells. The latter show eccentric nuclei and chromatolysis. In some cases only the nuclei are to be seen surrounded by small mononuclear cells. Glial proliferation is quite distinct.

Upper cervical cord.—In the meninges nothing abnormal made out. Ganglion cells mostly in good condition with granules well marked.

Central cervical.—As above, but a few small hæmorrhages present in both horns.

Upper and lower thoracic.—Nothing abnormal seen.

Lumbar cord.—Vessels congested.

Sacral cord.—Many degenerate ganglion cells in both horns with close application of proliferated glial cells. In certain instances as many as six to eight such nuclei could be counted.

Acute inflammatory changes were therefore observed within the nervous system. The lesions consisted chiefly of glial proliferation, slight perivascular infiltrations of glial cells and lymphocytes. In addition there were congestion, hyaline thrombi and multiple hæmorrhages into the perivascular sheaths and surrounding tissue.

The degenerative changes affected mainly the ganglion cells, and consisted of chromatolysis, vacuolation of the protoplasm and displacing of the nucleus. Other cells again were shrunken and stained deeply. The degeneration of these cells was such as to suggest that it had occurred during the life of the animal. In fact the short time that was allowed to elapse between the death of the animal and the fixing of the tissues almost excludes the possibility of any *post-mortem* change.

The general histological appearance shows a close similarity to those found in the first successfully inoculated monkey. One or two minor differences were observed, but they were rather of degree than of kind. The changes, on the whole, appeared to be earlier and therefore tended to be more general, in that the inflammatory lesions, although in relation to the blood-vessels, seldom consisted of dense perivascular accumulations. In addition, degeneration changes in the ganglion cells of the cord, particularly in the lower parts, were much more apparent. The affection of the cord, although in no way comparable to that found in experimental poliomyelitis, is of considerable interest. But before one can make definite statements on the relationship of this disease to poliomyelitis, a lot of work will have to be done. So far all experiments, serological and otherwise, tend to prove that the two viruses are distinct.

PASSAGE II.

Pieces of the spinal cord and brain (including basal ganglia) obtained at the autopsy of the *Macacus cynomolgus* monkey No. 1 were directly emulsified in normal saline, and inoculated under the dura and subcutaneously into two monkeys and three rabbits. The following results were observed :

A. Young Male Baboon.

21/4/20.—Inoculation.

22/4/20.—Quite recovered from operation.

26/4/20.—Quite fit.

29/4/20.—Ditto.

1/5/20.—Appears to be out of sorts, hair roughened and animal jumpy.

The above condition persisted for a week and then passed off.

15/8/20.—Animal noticed to be out of sorts. It gradually became weaker. The arms were noticed to be tremulous, and it had difficulty in picking things up with its fingers. No distinct paralysis was observed. The animal was described as dull and apathetic.

1/9/20.—In the afternoon the monkey had a well-marked fit which gradually passed off. Animal gradually became weaker and died after a fit on September 3rd.

Autopsy.—The autopsy was performed by Dr. Woods, who found nothing apart from some wasting of the tissues. Pieces of the brain and cord were placed in 50 per cent. glycerine for inoculation purposes, and the remaining portions were preserved for histological examination.

B. *Cercopithecus callitrichus.*

21/4/20.—Inoculated, but never showed the least abnormal sign, although kept under observation for over four months.

C. Rabbits.

21/4/20.—Three rabbits inoculated ; each received 0·25 c.c. intra-cerebrally and 2·5 c.c. subcutaneously.

22/4/20.—Animals fit and eat well.

23/4/20.—Ditto.

24/4/20.—Black rabbit rotates head from side to side.

26/4/20.—Black and grey rabbits out of sorts and stumbling in their gait. Temperature, brown 103·4° F., black 105·5° F. Both are very excited when touched.

27/4/20.—Brown rabbit has well-marked choreiform movements.

29/4/20.—Black rabbit very ill, more or less paretic, respiration slowed. Animal killed.

Autopsy.—Autopsy showed no macroscopic changes in the brain or internal organs. The cerebro-spinal fluid was clear and contained only a few lymphocytes. Pieces of the brain and spinal cord were fixed in formalin for histological purposes, and other portions were emulsified in saline and inoculated intra-cerebrally into three other rabbits, one of which developed similar symptoms.

The choreiform movements in the brown rabbit persisted for some time and then more or less completely passed off. The third rabbit never showed any abnormal signs.

Histological Changes in Rabbit's Brain and Cord.

Section through fore-brain shows a slight infiltration of the meninges with lymphocytes. In the cortex one or two small cellular infiltrations are seen. The ganglion cells seem mostly normal.

Section through mid-brain, including basal ganglia.—Meninges show a slight increase of lymphocytes. Inflammatory foci in the brain-substance not infrequent, consisting of mononuclear cells—lymphocytes and glial cells. The small vessels frequently have an infiltration of small cells along the sheath.

Upper cervical cord.—Nothing abnormal seen.

SPONTANEOUS INFECTION IN A *MACACUS CYNOMOLGUS.*

It is interesting to record here that a *Macacus cynomolgus* monkey kept as a control along with an inoculated monkey developed symptoms identical with those which the inoculated animal showed.

Three monkeys (*Macacus cynomolgus*) were obtained at the same time. On November 28th, 1919, two of these (a large male and a small female) were inoculated with the virus of the first passage. The third animal, a small male, was kept as the control. As neither of the two inoculated animals showed any sign of illness they were re-inoculated with the same virus on February 12th, 1920, the small female later on developing the infection in a typical manner. On February 14th, 1920, the small uninoculated male was observed to have a fit, although previous to this he seemed quite healthy. The animal then gradually became weaker, had one or two more fits, and died on February 21st, 1920. At the autopsy the brain was found to be slightly congested, but the other internal organs were quite healthy and nothing to account for death was detected.

A histological examination of the basal ganglia of the brain demonstrated the presence of inflammatory lesions such as we have become accustomed to consider as characteristic of *encephalitis lethargica*. These consisted of perivascular accumulation of lymphocytes and glial cells.

The fits, the absence of any gross lesion to account for the death of the animal, along with the characteristic histological changes found in the brain, justify one in regarding this as a spontaneous infection of encephalitis in a monkey. The period of time which elapsed between the death of the animal and the first inoculation of the companion monkeys was eighty-five days; it is quite likely, however, that the animals are infectious some considerable time before any symptoms develop as is the case with poliomyelitis. Owing to the impossibility of obtaining other monkeys at this period, inoculation experiments were not carried out.

CONCLUSIONS.

The successful transmission in series of experimental *encephalitis lethargica* to monkeys and rabbits has completed the experimental proof necessary to show that the disease is caused by a living virus. A similar result has been obtained by Levaditi and Harvier (2). The spontaneous case recorded above is additional proof. In the second passage the inflammatory lesion was not quite so strictly localised. This is apparently due either to some change in the virus or to the lesion not being so advanced. The greater involvement of the cord is in keeping with the more diffuse nature of the brain lesion.

In conclusion, I wish to express my indebtedness to Dr. Turnbull and to Dr. W. W. Woods for their valuable assistance in many directions.

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THE BRITISH JOURNAL

OF

EXPERIMENTAL PATHOLOGY

THE ÆTIOLOGY OF EPIDEMIC INFLUENZA: EXPERIMENTS IN SEARCH OF A FILTER-PASSING VIRUS.

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THE severity and wide distribution of epidemic influenza during the past two years has resulted in much discussion as to the ætiology of the disease, and has directed a large amount of effort towards the elucidation of that problem. From some areas the reported bacteriological findings[*] (1, 2) in clinical and post-mortem material have been interpreted as supporting Pfeiffer's belief that *B. influenzæ* is the causal agent. In other places this organism has been found in a much smaller proportion of cases (3, 4), and the discrepancy has been enough to cast some doubt upon the theory that it is the primary factor. That the differences in findings were not altogether due to differences in *personnel* and technique is indicated by comparing results, obtained during the epidemic in Toronto in the spring of this year, with those of the same men using the same technique working in England during the previous year. *B. influenzæ* was almost constantly present in the cases in England, but was found in only 24 per cent. of similar cases during the 1920 outbreak in Toronto. A glance over the recent literature will show that the pneumoccocus, the various streptococci and other bacteria varied in a similar way, and it therefore seems reasonable to regard these organisms, including *B. influenzæ*, as secondary invaders which vary in predominance in different localities, and in the same locality at different times.

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[*] A complete critical review of the literature upon the ætiology of influenza by Fildes and McIntosh appears in this Journal, vol. i, pp. 119 and 159.

Rosenow ([5, 6]) claims to have isolated from cases of influenza a green-producing streptococcus which has pandemic characteristics, and as a result of animal experiments he draws certain analogies between the lesions produced by this organism and the pathological findings in human influenza. His work has not yet been confirmed by other observers.

It is perhaps fair to state that the trend of opinion has gradually been in favour of the theory that the primary infecting agent is a filter-passer.

Keegan ([7]), however, reports a complete absence of symptoms in nine volunteers who were inoculated with filtered sputum and nasal washings from influenza patients. Two patients were used, one in the second and one in the fourth day of the disease. The nasal washings (75 c.c. from each) were mixed with the sputum, shaken and then filtered. Half a cubic centimetre of the filtrate was sprayed into the nose of each volunteer, four being used for one patient and five for the other. They were isolated for ten days and had no symptoms.

Rosenau ([8]), in extensive experiments, carried out on a large number of volunteers, found that no symptoms occurred in the subjects after they had been inoculated with large amounts of nasopharyngeal and bronchial secretions, or with whole blood, from cases of typical influenza. Similar inoculations with suspensions of Pfeiffer's bacillus also failed to produce any symptoms.

Nicolle and Lebailly ([9]) report symptoms of influenza in two volunteers who had been inoculated subcutaneously with filtered sputum from influenza patients; also in two monkeys inoculated under the conjunctiva and by nasal instillation with the same sputum unfiltered.

Dugarric de la Rivière ([10]) caused himself to be inoculated subcutaneously with filtered blood from influenza patients, and as a result became ill with the typical symptoms of influenza. He considered that this afforded him protection, as no symptoms resulted from spraying his throat and nasopharynx with filtered influenzal sputum on the tenth day after the first inoculation.

Yamanouchi, Sakakami and Iwashima ([11]), by nasopharyngeal inoculation of volunteers with influenzal sputum, both filtered and unfiltered, produced typical symptoms of the disease in eighteen out of twenty-four subjects. The six failures were the only ones in the series who had previously had influenza, and it was therefore presumed that they had immunity. Filtered blood from influenza patients, administered in the same way, and subcutaneous inoculation of filtrates from blood or sputum, produced similar results. A pure culture of Pfeiffer's bacillus and mixed cultures of pneumococci streptococci, staphylococci and the cocci common in sputum sprayed into the throat and nose of fourteen healthy people did not cause any illness.

Olitsky and Gates ([12]), by intratracheal injection of filtered bronchial and nasopharyngeal secretions from influenza cases during the first thirty-six hours of the disease, produced in rabbits a condition resembling the pathological change of influenza in man.

Further evidence in support of this view has been furnished by Bradford, Bashford and Wilson ([13]) and Gibson, Bowman and Connor ([14]). The former describe a minute, filter-passing organism which they found constantly in cases of influenza, and which they were able to grow by special cultural methods. After inoculation with subcultures they record symptoms and lesions in monkeys, and especially in guinea-pigs, which are noted as typical of the disease in man. The organisms were recovered in pure culture from the animals.

Gibson, Bowman and Connor independently isolated an organism which both groups of workers considered identical with that described by Wilson. They found "typical experimental influenzal lesions" in monkeys, rabbits, guinea-pigs and mice after inoculation with cultures, and recovered the organism from the tissues of the animals. Earlier in their work, before they had obtained cultures, the same typical lesions were seen in animals after the introduction of blood and sputum from influenza patients, and, by reinoculation of filtered extracts from abnormal experimental lungs, the same pathological change was repeated in rabbits and guinea-pigs up to the second reinoculation.

It seemed to us desirable that further evidence should be obtained as to the ætiological relationship of a filter-passing organism to influenza, and this investigation was commenced with that object in view.

OBSERVATIONS ON THE PATHOGENICITY OF INFLUENZAL FILTRATES.

The patients from whom we obtained our material were all typical cases of influenza from the epidemic of 1919–1920. They were in the wards of the service of Dr. Duncan Graham, Professor of Medicine, and for the most part were in the earlier stages of the disease.

The various materials obtained from these cases during life or after death were filtered through thoroughly tested Berkefeld or Mandler filters. Such filtrates were always bacteriologically tested.

Guinea-pigs were largely used for the inoculations by the intra-peritoneal route. Rabbits and mice were used less often. All animals were kept under the best hygienic conditions and were killed for autopsy by a sharp blow on the back of the neck.

In general it was found that all animals examined a few days after inoculation with these products showed marked hæmorrhagic lesions in the lungs but no gross changes in other organs. A filtrate prepared from such lungs or from the blood of the same animal reproduced the same lesions in a second animal, and, indeed, these could be carried through eight passages.

Findings in Inoculated Guinea-pigs.

Since most of the work was done with guinea-pigs, it will be necessary to discuss in some detail the findings after inoculating these animals. We have already mentioned the fact that when they were killed a few days after injection, hæmorrhagic lesions in the lungs were found in a considerable number.

The first striking feature noted about the animals was that they did not become ill. The temperature of all our inoculated animals was carefully taken for a time and no abnormalities found; and it soon became apparent that we could not judge the probability of pulmonary lesions from watching the behaviour of the pig.

The essential feature of the typical lesion was the peculiar type of hæmorrhagic change. This was patchy in distribution, it extended into the lung substance and could be seen on section. On the surface it appeared in varying forms. Sometimes the whole lung was almost covered with small red areas 2 to 3 mm. in diameter. Sometimes these were more sparsely scattered, or they might be confined to one or two lobes. The larger lesions appeared to result from coalescence of the smaller ones, and were often 10 to 15 mm. in diameter. The centre of such an area was usually a dark, deep red colour, and the periphery, diffuse and irregular in outline, was a brighter red. Sometimes a large part of one lobe was solidly involved in this change, and when this was true it was frequently the part nearest the root of the lung. There was never any fluid exudate or fibrin in the pleural cavity.

A few flecks of red on the surface was not considered abnormal. Neither was a greyish, glistening, slightly raised appearance that had a small and irregular distribution, nor small, pale, slightly raised areas that occurred irregularly. These were found frequently in pigs killed for other purposes.

Two other more extensive atypical lesions were also seen, sometimes in pigs with no previous inoculation, as well as in inoculated animals with or without hæmorrhagic change. They were considered to have no connection with the typical change. In one sort the lung was hard and the surface uneven. It was a yellowish grey colour, and hard yellowish nodules 2 to 3 mm. in diameter showed through the surface. Usually a whole lobe was involved, frequently an upper one, and sometimes two or three lobes were affected. Microscopically it was seen to be a chronic inflammatory change. The air-space was completely obliterated, and the picture was that of the organisation of a previous pneumonic condition.

The lung of the other type was smaller in size than normal. It appeared solid and homogeneous and had a dull salmon-pink colour. Frequently the small posterior lobe was alone affected, sometimes small parts of two or three lobes near the hilum, or perhaps only an irregularly shaped part of one lobe. The gross appearance gave one the impression of a congenital atelectasis.

The cultures of the organs made at the autopsy were in most cases sterile. However, a small proportion of the hæmorrhagic lungs gave positive cultures, the predominating organisms being small Gram-negative bacilli that did not produce gas from glucose and streptococci. *B. coli* was found in a few cultures from abdominal organs.

The filtrates, with very few exceptions, were sterile. The failures were not due to faulty filters, but probably to contamination from the air in handling the filtrate. A large Gram-positive bacillus and a large Gram-negative bacillus were the only organisms found. These were evidently not responsible for the lesions in the inoculated animals, for such contaminations were very rare (as may be seen from the protocols cited), and the same lesions were present when the inoculant was sterile.

Nor were the hæmorrhages due to the protein injected. The animals had never been treated before; they were not sensitised; they did not exhibit any anaphylactic symptoms, and the appearance of the lung was not that of a protein lesion ([15]). Gross changes in the other organs were very uncommon and are described later. The main interest centred in the typical pulmonary lesions, and the following table shows the incidence of animals in which they were found and the inoculant associated with them.

TABLE I.—*Showing the Numbers of Guinea-pigs Inoculated and the Number that had Pulmonary Lesions.*

Inoculant used.	Number of animals inoculated.	Number that showed gross pulmonary lesions.	Number with no gross lesion in the lungs.
Patient's whole blood	31	10	21
Patient's filtered red blood-corpuscles	27	17	10
Patient's filtered plasma	18	8	10
Filtered empyema fluid	4	4	0
Patient's filtered nasal washings	2	0	2
Filtered human tracheal mucous membrane	2	1	1
Filtered positive guinea-pig lung	66	35	31
Filtered blood from positive guinea-pig	11	6	5
Whole blood of positive guinea-pig	43	25	18
Laked blood of positive guinea-pig	6	2	4
Unfiltered lung of positive guinea-pig	15	8	7
Unfiltered lung of negative guinea-pig	3	1	2
Filtered lung of negative guinea-pig	12	3	9
Whole blood of negative guinea-pig	11	8	3
Filtered influenzal sputum	14	9	5
Unfiltered influenzal sputum	2	2	0
Totals	267	139	128

After one type of inoculant only did we fail to find pulmonary hæmorrhages, viz. filtered nasal washings. But the number of animals used in this instance was so small that no conclusion can be drawn. If more had been inoculated we probably would have found hæmorrhages in some. Typical hæmorrhages were present after patients' whole blood, sputum and filtrates from blood-corpuscles, plasma, empyema fluid and tracheal mucous membrane. Considered altogether, approximately half of the guinea-pigs had typical lesions.

Reviewing the results in animals inoculated directly from each of the seventeen cases, it is seen that some guinea-pig lesions were associated with every patient except one, and that approximately half of the total number of pigs inoculated were affected. The following table shows this in detail:

Table II.—*Showing the Numbers of Guinea-pigs Inoculated from the various Influenza Cases and the Number that had Pulmonary Lesions.*

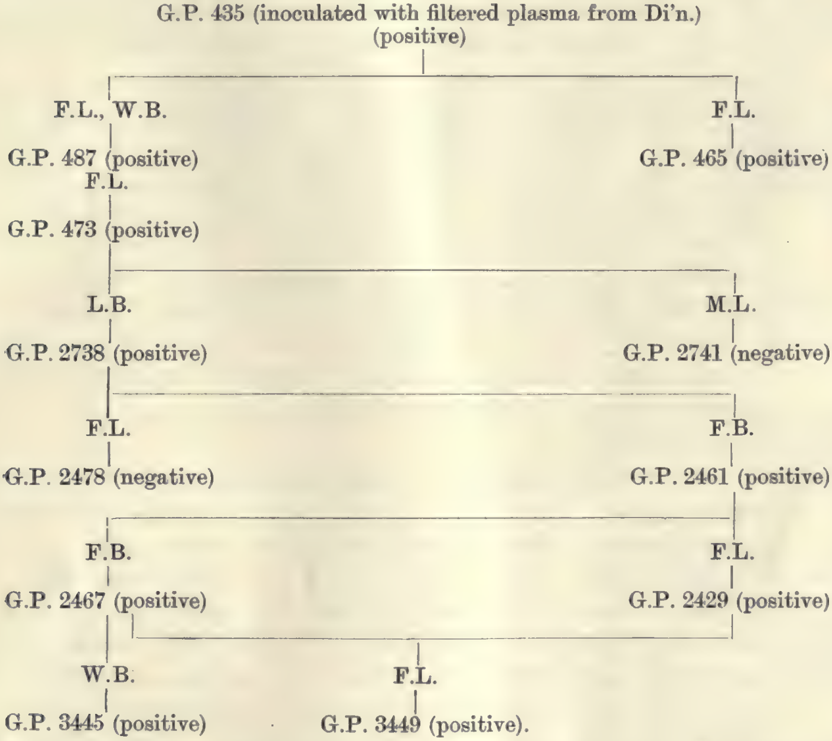
Name of patient.	No. of animals inoculated.	No. positive.	No. negative.
Ba'd.	14	5	9
Ba't.	4	2	2
Be'l.	4	1	3
Di'n.	13	9	4
Gr'n.	2	2	0
Gr'g.	3	3	0
Ki'e.	6	2	4
Le'l.	4	2	2
Ma's.	4	2	2
Ra'n.	17	11	6
Ro'y.	4	0	4
Sh'i.	4	2	2
Sh'n.	2	2	0
Autopsy.	1	0	1
Is'd.	7	2	5
Ro'n.	4	2	2
Co's.	5	4	1
Ma'n.	2	2	0
Totals	100	53	47

After reinoculation the same pathological picture appeared for several repetitions. In one instance it was found after seven reinoculations, once after six, once after five, and twice after three. From our subsequent experience, it seems certain that by using large series of animals this repetition of lesions could have been carried on indefinitely. In only two series did reinoculation fail completely. Frequently both the blood and lung of one animal were used to inoculate two, and we therefore sometimes had four or five animals an equidistant number of reinoculations removed from the one patient. As a rule, the more animals used each time the more chance there was of continuing, for if one failed to show hæmorrhages another might do so. There was never an element of surety that reinjection of a pathological lung would reproduce the same picture in the next animal. Sometimes blood was successful, sometimes lung, and sometimes both failed.

The length of time the animal was allowed to live did not seem to be a factor, for typical lesions were found as early as two and as late as nineteen days.

The following chart illustrates a typical series of reinoculations in guinea-pigs:

CHART 1.



W.B. = whole blood. L.B. = laked blood. F.B. = filtered blood. F.L. = filtered lung. M.L. = mashed lung.

The following are the protocols relating to Chart I.

PROTOCOL I.

19:2:20. G.P. 435. Inoculated with 3 c.c. filtered plasma from Di'n. Filtrate sterile. No sign of illness. Killed 4 days after injection. Lung: many bright red hæmorrhagic areas. Heart's blood, lung, liver, spleen: sterile.

23 : 2 : 20. G.P. 487. Inoculated with 3 c.c. whole blood of G.P. 435. No sign of illness. Killed 3 days after injection. Lungs: marked and very numerous hæmorrhagic areas throughout. Lungs "stand up" and are speckled with these small hæmorrhages, from 1 to 3 mm. in diameter, and a few larger. Heart's blood, lung, liver, spleen: sterile.

23 : 2 : 20. G.P. 465. Inoculated with filtered lung from G.P. 435. Filtrate sterile. No sign of illness. Killed 4 days after injection. Lungs mottled with brownish dull red areas and pale raised patches. These areas vary much in size. Spleen slightly enlarged. Heart's blood, spleen, liver: sterile. Lung: large Gram-positive bacillus.

26 : 2 : 20. G.P. 473. Inoculated with one-half of filtrate from lung of G.P. 487. Filtrate sterile. No sign of illness. Killed 5 days after injection. Lung: numerous reddish-brown hæmorrhagic areas with dark centre and diffuse red periphery. Heart's blood sterile. Lung, liver, spleen: Gram-positive cocci in pairs and chains, non-hæmolytic.

2 : 3 : 20. G.P. 2738. Inoculated with 3 c.c. whole blood from G.P. 473 + 7 c.c. distilled water. No sign of illness. Killed 5 days after injection. Lung: thickly spotted with areas of hæmorrhagic change, 3 to 6 mm. in diameter, seen on surface and on section. Liver: deeply congested. Heart's blood, lung, liver, spleen: sterile.

2 : 3 : 20. G.P. 2741. Inoculated with lung of G.P. 473 mashed in sterile distilled water. No sign of illness. Killed 5 days after injection. No gross lesions. Heart's blood, lung, spleen, liver: small Gram-positive bacillus.

2 : 3 : 20. G.P. 2478. Inoculated with filtered lung from G.P. 2738. Filtrate sterile. No sign of illness. Killed 6 days after injection. No gross lesions. Heart's blood, lung, liver, spleen: sterile.

7 : 3 : 20. G.P. 2461. Inoculated with 3 c.c. blood from G.P. 2738 laked and filtered. Filtrate sterile. No sign of illness. Killed 7 days after injection. Lung: thickly spotted with areas of hæmorrhage 3 to 4 mm. in diameter. Some are confluent and form larger areas; seen on section and on surface. Spleen slightly large. One hæmorrhagic area, 4 × 6 mm., on large bowel. Heart's blood, lung, liver, spleen: sterile.

14 : 3 : 20. G.P. 2467. Inoculated with 4 c.c. blood from G.P. 2461 laked and filtered. Filtrate sterile. No sign of illness. Killed 7 days after injection. Lungs: on two lobes there are hæmorrhagic areas, about 1 cm. in diameter, with dark red centre and diffuse brighter red periphery. Heart's blood, lung, liver, spleen: sterile.

14 : 3 : 20. G.P. 2429. Inoculated with filtered lung from G.P. 2461. Filtrate sterile. No sign of illness. Killed 7 days after injection. Two or three small red flecks on surface; not considered patho-

logical. No other gross change. Heart's blood, lung, liver, spleen: sterile.

21 : 3 : 20. G.P. 3445. Inoculated with 5 c.c. whole blood from G.P. 2467. No sign of illness. Killed 8 days after injection. Lungs: few small hæmorrhagic areas scattered throughout. No other gross lesions. Heart's blood, lung, liver, spleen: sterile.

21 : 3 : 20. G.P. 3449. Inoculated with filtered lungs of G.P. 2467 and G.P. 2429. Filtrate sterile. No sign of illness. Killed 8 days after injection. Lungs: three hæmorrhagic areas extending into the lung substance. Heart's blood, lung, liver, spleen: sterile.

Charts 2 and 3 with the accompanying protocols show similar results and have been omitted.

Looking for corroborative evidence of the infective nature of the inoculant, on two occasions we heated the filtered plasma and filtered corpuscles at 56° C. for one hour before injecting them, and at the same time inoculated an equal number of animals with unheated filtrates from the same patient. The following table shows the comparison of the results from each:

TABLE III.—*Showing the Results from Injecting Heated and Unheated Filtrates.*

Name.	Inoculant.	Unheated.			Heated.		
		Number of animals.	Number positive.	Number negative.	Number of animals.	Number positive.	Number negative.
Be'l.	Filtered plasma	2	1 (10 days)	1 (7 days)	2	1 (8 days) 1 (9 days)	0
	Filtered corpuscles	2	0	1 (7 days) 1 (10 days)	2	0	1 (8 days) 1 (9 days)
Sh'i.	Filtered plasma	2	0	1 (8 days) 1 (8 days)	2	1 (8 days)	1 (10 days)
	Filtered corpuscles	2	1 (19 days) 1 (19 days)	0	2	1 (8 days) 1 (19 days)	0
Totals .		8	3	5	8	5	3

It is certain that heating the inoculant to the extent noted did not diminish the lesions. In fact more hæmorrhages occurred after heating, which indicates that some pigs with heated filtrate had lesions when those with corresponding unheated filtrate had none.

Plasma from three convalescent patients was tested for any power it might possess to inhibit the lesions found after inoculation of filtrates.

The following is a brief note of the patients from whom they were obtained:

A. (Ra'n.). Patient became ill February 12th, 1920; had high fever and consolidation of lung. Temperature continuously normal from February 20th, 1920. Blood removed on March 5th after patient had left hospital.

B. (He'y.). Patient became ill March 8th, 1920; had fever, but no pulmonary consolidation; was markedly ill. Temperature continuously normal March 15th. Blood taken March 18th, 1920.

C. (M. Sm'h.) First ill March 1st, 1920; had pulmonary consolidation and severe illness. Temperature continuously normal March 16th. Blood taken March 18th.

The plasma from these three samples of blood was filtered and mixed with filtrates from pathological animals in amounts varying from 3 to 5 c.c. These mixtures were incubated at 37° C. for one hour (air) before inoculation.

Control injections of the same filtrates, without plasma, and the convalescent plasma alone were always made at the same time.

Altogether eleven guinea-pigs were inoculated, and the results failed to show that the convalescent plasma had any effect in preventing the pulmonary lesions. In one instance hæmorrhages were seen after inoculation with a filtrate from a hæmorrhagic lung incubated with plasma, while the animal injected with the same amount of filtrate alone had none.

Furthermore, convalescent serum was incubated with an influenzal patient's whole blood for one hour and injected subcutaneously into two guinea-pigs (in amounts of 5 c.c.), one of which was killed in four days and showed a few scattered hæmorrhages throughout both lungs. The other was killed in thirty-one days, and in its lungs were found numerous fairly large consolidated areas, which proved on microscopic examination to be of the proliferative type.

The gross lesions in other organs were very infrequent, being noted only six times in a series of 159 animals. They were in detail as follows: hæmorrhage into wall of bowel, twice; slight hæmorrhage on surface of liver, twice; hæmorrhage 8 × 10 mm. into rectus abdominis muscle, once; extensive retroperitoneal hæmorrhage in lower abdomen, once. The spleen was sometimes slightly enlarged. These occurred so seldom that their significance was not regarded as important when compared with the frequency of pulmonary hæmorrhage, which was undoubtedly the typical lesion of the condition with which we were dealing.

Both the organs with gross lesions and those apparently normal were studied microscopically.

Lung.—The microscopic changes throughout the series were of two types —hæmorrhagic and proliferative. The former corresponded to the gross and so-called "typical" lesion; the latter was not always apparent to the naked eye. These may for convenience be described separately, although they sometimes occurred together.

The proliferative lesion will be considered first. In very many sections the alveolar walls were thickened, due to proliferation of endothelial cells. Where the change was least the outline of the alveoli could easily be traced, but the air-space of the lung was considerably lessened. In places of greater change single alveoli were completely obliterated by the proliferation; others near by were only a small fraction of their original size, and the outline of the alveolar walls was lost in the diffuse mass of cells. Where this was most marked the tissue was solid and the air-space entirely obliterated. The cells were rather large and irregular in outline; the nuclei were large, central, oval or round, and stained with moderate intensity. The cell masses included capillaries. Giant-cells were occasionally seen. A recent thrombosis of a small branch of a pulmonary artery was associated with this type of lesion.

Although some lungs showed only proliferation, it was common to find hæmorrhage as well. Its situation was variable. It was sometimes small in amount and irregular in distribution, situated interstitially in the thickened alveolar wall or in a mass of cells; but it was also extensive in some areas, filling the partially obliterated alveoli and infiltrating the interstitial tissue between them.

The picture presented by this combination did not suggest that the two processes had a common cause, or that one resulted from the other. Both the hæmorrhage and the proliferation occurred alone so definitely and so frequently that their association appeared to be accidental, and could not be construed as signifying a more fundamental relationship. Further, where they did occur together, in no instance was hæmosiderin present in the endothelial cells. This fact strongly confirms the view that the lesions were independent of each other.

The hæmorrhage was undoubtedly the most singular and striking lesion. It was this that in the gross attracted one's attention, and was described in the articles (13, 14) already mentioned as typical of "experimental influenza" in animals, and closely comparable to the early lesions of influenza in the human lung. After reading these accounts we were satisfied that the hæmorrhages we had seen were the same lesions that they reported, and in fact one of the workers already quoted (14), having examined our specimens in our laboratory, agreed in this opinion.

The amount of lung involved in the hæmorrhage varied considerably, as may be judged from the description of the gross appearance. The minutest lesion was a capillary hæmorrhage into the alveolar wall, but it was always just an incident in a more extensive change. In most sections the hæmorrhage appeared in small patches. In these the alveoli were completely filled with blood and stood out sharply defined from air-holding tissue. Where they coalesced to form a massive hæmorrhage large sections of the lung were airless, except for a few scattered empty alveoli that were prominent because of contrast. In many sections blood was seen in the bronchi, and in a very few places slight œdema was observed. A peculiar feature was the lack of leucocytic reaction. Several times there was a recent thrombosis of a branch of the pulmonary artery. The endothelial proliferation, as we have pointed out, could not be regarded as a response to the extravasated blood. Neither in the hæmorrhage itself, nor in the alveolar walls that trabeculated it nor around the margin of the area was any excess of leucocytes seen. The thrombi, however, did contain a moderate number of polymorphonuclear leucocytes.

The thick and irregular muscular coat of the arteries, the folded epithelium of the bronchioles and the masses of lymphocytic cells in the peribronchial tissue are normal structures in the guinea-pig's lungs.

The microscopic lesions in other organs were not frequent, but the following were noted:

Myocardium.—Congestion, without any degeneration or œdema. Slight round-cell infiltration about some of the smaller arteries. Several small diffuse hæmorrhages. The muscle-fibres were broken in these areas, but there was no leucocyte reaction.

Kidney.—Varying degrees of congestion, sometimes interstitial, sometimes affecting the glomeruli only. There was no degeneration nor exudate in the tubules. In one case small areas of interstitial œdema were present alongside the focal interstitial congestion.

Liver.—Marked congestion was occasionally seen. Associated with this small focal hæmorrhages were present in one instance, with diffuse œdema in the portal fibrous tissue, but complete absence of leucocyte reaction.

Spleen.—A few sections show congestion.

Findings in Inoculated Mice.

Fifty-six white mice were inoculated intraperitoneally—with the exception of a few subcutaneously—with material from six cases of influenza. The plan of procedure and technique outlined above for guinea-pigs was followed for these animals. Although some mice had hæmorrhagic lungs we considered it impossible to deduce any experimental evidence from the findings, because a very large percentage of animals yielded cultures of ordinary bacteria. It was common to find organisms from the heart's blood, and the other organs were very frequently infected. The flora of these cultures was not studied fully.

Findings in Inoculated Rabbits.

Six rabbits were inoculated intravenously and one intraperitoneally with filtrates of plasma or corpuscles from four influenza patients. Of these, four had no gross lesions, one had a few small hæmorrhages in the lungs, one had a small hæmorrhage into the tracheal mucous membrane, and one hæmorrhage into the trachea and œdematous and congested lungs. Fourteen cubic centimetres of the whole blood of this rabbit was injected intravenously into another. It showed one small hæmorrhage in the lung. There were no other gross lesions. None of the animals were ill.

The microscopic study of the lungs showed lesions strictly similar to those described for guinea-pigs. There was both endothelial proliferation and hæmorrhage. The latter was not so massive as in guinea-pigs and more œdema was present, although this was not a prominent feature.

OBSERVATIONS ON THE CULTURE OF A FILTER-PASSING ORGANISM.

An attempt to cultivate a "virus" from filtrates was made by employing a special technique.

A small piece of fresh, sterile rabbit's kidney was put in the bottom of a test-tube (1·5 × 15 cm.) and sterile ascitic fluid added to the depth of about 3 cm. This was incubated for twenty-four hours to ensure sterility, and was stored in the ice-chest until used. Tubes a week old were discarded. After inoculation more ascitic fluid was added until the column was 8 to 9 cm. high. On top of this was poured in about 1 cm. of melted vaseline, which had been previously sterilised. Incubation was carried on at 37·5° C.

In this manner we cultured filtered plasma and filtered corpuscles from three cases (Ba't., Ro'y. and Ki'e.), filtered hæmorrhagic lungs from four guinea-pigs, and filtered nasal washings from one patient (Ki'e.). Each was inoculated into three different samples of ascitic fluid and thorough controls were made.

The cultural appearances of filtered corpuscles were constant and differed from the others. In twenty-four to forty-eight hours a faint diffuse cloudiness appeared in the lower 2 cm. of the tube. This gradually spread throughout the fluid, and was followed by a general increase in opacity. In two to four days a very finely granular greyish deposit appeared in a streak along the side of the tube and to a slight extent in the bottom. This was very noticeable because the quantity was so large.

The other cultures became faintly cloudy in the lower third of the tube in three to five days. Sometimes this became diffuse, sometimes a very finely granular deposit appeared in small amounts about the kidney tissue or for a short distance up the sides of the tube.

As far as we could determine, these appearances did not indicate the growth of an organism, for tubes inoculated with filtered corpuscles and filtered plasma from one of us who had never had influenza changed in exactly the same way. Moreover films of the cultures, fixed in methyl alcohol and stained for fourteen to eighteen hours with Giemsa's stain, and also with Gram's stain, did not reveal the presence of any organisms.

Examinations were made up to the end of four weeks.

It was, however, decided to inject some of the cultures into animals, and the following are the results of this procedure:

Two guinea-pigs, 3747 and 3733, were each given intraperitoneally 3 c.c. of a three-day-old culture of filtered corpuscles from Ba't. After six days no typical lesions were found. Pigs 7477 and 7461 were similarly inoculated with 3 c.c. and 2 c.c. respectively of a five-days-old culture of filtered corpuscles from Ki'e. Both were killed on the fifth day and had no gross lesions.

A five-days-old culture of filtered corpuscles from Ro'y. was used on two pigs—No. 7444 was given 2 c.c. and No. 7488 2·5 c.c. The former, killed after five days, had extensive hæmorrhagic change in the lungs. The filtered lung was injected into pig No. 3732, which was killed after five days and had no gross lesions. Pig No. 7488 was normal at the end of forty-eight hours.

One pig was inoculated with a twenty-three-days-old culture of a filtrate from a hæmorrhagic guinea-pig's lung. It was killed nineteen days afterwards and showed marked pulmonary hæmorrhage.

These results are very far from indicating the causal agent in influenza, and in itself this cultural work is far too meagre to exclude a filter-passing organism as the ætiological factor. We wish, however, to record the technique, and to point out that in two animals the typical pulmonary lesion was found at five and nineteen days after inoculation with apparently sterile cultures.

That this lesion was due to some invisible virus in the culture was not an impossible view, but it seemed a very improbable one. This attitude was further strengthened by the results observed after ordinary broth cultures of filtrates were injected into animals.

The following table summarises these results:

TABLE IV.—*Summary of Results from Inoculation of Broth-cultures of Filtrates.*

Inoculant.	Name.	Number of guinea-pigs inoculated.	Number positive.	Number negative.
12-day culture of filtrate from hæmorrhagic guinea-pig lung	Di'n.	1	1 (8 days)	—
23-day ditto	Le'l.	1	—	1 (19 days)
48-day „	Ra'n.	1	—	1 (7 days)
8-day „	Sh'n.	1	1 (6 days)	—
28-day culture of filtered blood from a positive guinea-pig	—	—	—	1 (9 days)

These cultures were those made as a routine test of sterility of all our filtrates. The older ones had been purposely kept in the incubator in order to correspond to the long incubation usually associated with filterable organisms, but we were unable to demonstrate organisms of any sort in them. Inoculation was intra-peritoneal.

Two animals had typical lung lesions. The filtered lungs of each of them were transferred to two other pigs, and these showed more marked lesions than the first pair—in thirteen days and eight days respectively.

The pathological changes in all of these "positive" animals was exactly the same as that already described.

If we regard it as due to some invisible "virus," we must consider this "virus" to have grown in two widely different types of culture, to have been active in cultures as young as five days and as old as twenty-three, and from different types of cultures to have produced the same sort of lesion in animals at five and twenty-nine days after inoculation. In view of these conditions it seems much more reasonable to believe that the lesions did not result from the injection of the cultures.

TABLE V.—*Showing the Results from Inoculating Guinea-pigs with Filtrates of Material from Healthy People.*

Name.	Diagnosis.	Inoculant.	Number of animals Inoculated.	Number positive.	Number negative
Sh'g. .	Fractured leg	Plasma	2	—	2
		Corpuscles	2	—	2
Br'n. .	Fractured arm	Plasma	2	1	1
		Corpuscles	2	1	1
Mo'n. .	Supra-orbital neuralgia	Plasma	1	—	1
		Corpuscles	3	3	—
Ca'e. .	Inguinal hernia	Plasma	2	—	2
		Corpuscles	2	—	2
Hi'g. .	Tuberculous adenitis of neck	Plasma	3	2	1
		Corpuscles	3	2	1
Ga'h. .	Injury to musculo-spiral nerves	Plasma	2	1	1
		Corpuscles	2	1	1
Mu'y. .	Injured perinæum	Plasma	3	2	1
		Corpuscles	2	1	1
H.B.M. .	Well	Plasma	2	1	1
		Corpuscles	1	—	1
Ra'u.* .	Well	Plasma	2	—	2
		Corpuscles	2	—	2
Wi'i. .	Asthmatic bronchitis	Laked whole blood	2	2	0
	Totals		40	17	23

* Blood removed just after convalescence from severe influenza, thirteen days after the temperature first became continuously normal.

CONTROL EXAMINATIONS OF HEALTHY PEOPLE.

As a matter of comparison we inoculated a series of guinea-pigs with filtrates from the corpuscles and plasma of healthy people. The technique was exactly the same as that used for influenza patients. The healthy controls were for the most part people in the surgical wards of the hospital, and none of them had ever had influenza, nor did they develop the disease within a reasonable time after our examination. The results are summarised in Table V, p. 277.

The character of the lesions in these animals agreed in every way with those found after injection of influenzal material. The positive animals shown in the table were those which had typical hæmorrhagic lung changes. Their number was almost half of the total inoculations. Comparing this with the similar table from influenza cases, and considering the small series on which this is based, the percentage of positive animals may be regarded as approximately the same, whether the material injected came from healthy people or influenza patients.

After adding this evidence to that obtained from inoculation with cultures, it was quite apparent that none of the animal lesions had any connection with the infective agent of influenza.

OTHER CONTROLS.

This conclusion was fully justified by the subsequent examination of guinea-pigs that had never received any inoculation.

Three lively and apparently healthy pigs, about 200 grm. weight, were chosen at random from the breeding-pens. Two were killed by a blow on the head and one by cutting the throat. All had very extensive and typical hæmorrhagic change.

The lungs of twenty-three pigs, bled for complement, were also examined. They had previously been used for testing diphtheria antitoxin but appeared healthy when killed. Three of them had hæmorrhages.

These findings revealed the fact that we were dealing with some pathological condition present in our stock pigs. Furthermore we found that not only were our animals affected but those from other laboratories also. Part of our supply came from a farm on the outskirts of the city, and in transit they were sometimes kept for a few days in another laboratory in another building. Four pigs, from a batch that came to us in this way, were killed by a blow on the head immediately they arrived. Two had the typical hæmorrhagic lesion.

DISCUSSION.

Neither the proliferative type of pneumonia nor the hæmorrhagic lesions herein described appear to have received notice in the literature upon spontoneous animal disease. The pneumonia in guinea-pigs attributed to infection with *B. bronchisepticus* by Theobald Smith (16) seems to have been an acute process, with necrosis of the parenchymal cells and infiltration of the area with both endothelial and polymorphonuclear cells. It is possible that the proliferative change we have found may be a late stage of the same disease, but this seems very unlikely. Smith described the involved area of lung in the late stage as "firm, greyish and shrunken to quite small dimensions." He also mentions that the earliest stages of the disease are not definitely known, and it may be that this is what we have observed.* Another possibility that suggests itself is that this proliferative change is due to the dust and particles of *débris* which must of necessity be breathed into the respiratory tract by the guinea-pig by reason of its environment in the ordinary methods of rearing these animals under the usual artificial conditions.

The typical hæmorrhagic lesions described also lack an adequate explanation at the present time. Hæmorrhages in various localities are frequently encountered by every observer experimenting upon animals by inoculating with pathogenic organisms. In such cases, however, one is never in doubt as to the meaning of such lesions, since not only is a typical cellular reaction observed about the area, according to the length of time elapsing after the inoculation and the localisation of the infecting agent, but even in the earliest stages microscopic sections through the area of hæmorrhage will reveal the actual presence of the bacteria in or about the capillaries involved. Moreover, appropriate cultural methods will recover the organism from the lesion.

None of these conditions obtained in the type of hæmorrhage under discussion, since the inoculants did not contain any of the known pathogenic bacteria. It would, however, seem reasonable to expect the usual reaction to infection about such areas if an infecting organism were the cause, and if sufficient time had elapsed for such processes to take place. In almost every instance the red blood cells appeared so fresh and took up oxygen so readily that one was led to think that the hæmorrhage was probably very recent. That it might have been due to the method of killing the animals has been considered. About 300 have been killed by a blow on the back of the head and approximately half of them have had the hæmorrhagic lesion. Records of twenty-four guinea-pigs killed by rapid bleeding from the jugular and carotid vessels show that four had hæmorrhages.

Further investigation is needed to settle the exact ætiology of these lesions, and the questions briefly discussed above will form the basis of a further communication in an attempt to throw some light upon them.

SUMMARY AND CONCLUSIONS.

There is considerable evidence in the recent literature for attributing epidemic influenza to a primary infection with a filter-passing organism. The most extensive experiments by animal inoculations have been carried out by Bradford, Bashford and Wilson, and Gibson, Bowman and Connor, who reported having found such an organism in tissues and exudates, and obtained it in culture. By inoculating animals, principally guinea-pigs, with filtrates of material from patients and with cultures, they produced "typical experimental influenzal lesions," and recovered the organism in culture from the animals. They also succeeded in transferring the typical lesions from one animal to another. Bradford and Wilson, in a note appended to a criticism by Arkwright (17), later retracted their claim that a filter-passing organism had been grown in pure culture, and stated that degeneration forms of bacteria and protein particles in their cultures had been mistaken for such an organism. This retraction may also be applied to the work of Gibson, Bowman and Connor, since they state that they were in agreement with Capt. Wilson that their "organism" was the same as he had found.

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* *B. bronchisepticus* was recovered from some of our apparently healthy animals killed for controls, and they may, as carriers, have developed some immunity towards this organism which would account for a low grade of tissue response to infection with it.

We have investigated, with a technique similar to theirs, seventeen cases of influenza, and by inoculating animals, principally guinea-pigs, with filtrates of material from these patients have observed the same typical lesions. By reinoculating one guinea-pig from another we have found the lesions repeated through seven reinoculations.

Heating the inoculant to 56° C. for one hour did not diminish the animal lesions, nor did incubation with convalescent plasma.

We were not able to obtain cultures of a filterable organism, although the attempt to do so was not exhaustive.

We did find, however, that the *typical* lesions appeared in guinea-pigs after inoculation with *sterile* cultures of widely different types and ages.

Further, this "typical" lung change was found in guinea-pigs from our stock and breeding-pens that had never been inoculated, and also in animals that came to us from a breeding-farm and another laboratory.

The same percentage of animal lesions occurred after inoculation with material from healthy controls as with influenzal material.

The lesions under discussion are microscopically of two broad types, proliferative and hæmorrhagic, and have been described in the papers previously quoted. We have pointed out, however, that these two types of tissue change apparently bear no fundamental relationship to each other, and were probably only accidentally associated. They were undoubtedly not produced by the infective agent of epidemic influenza.

The proliferative type is to be regarded as a spontaneous animal disease, the bacteriology of which is being investigated. The hæmorrhagic type of pulmonary lesion is peculiar, inasmuch as there is a striking absence of cellular or other reaction about the areas involved. Its ætiology is obscure, and further work is being done in an endeavour to find an adequate explanation of its occurrence.

The authors desire to acknowledge many helpful suggestions and criticisms from Prof. J. J. Mackenzie and Prof. Duncan Graham during the course of the work.

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THE EFFECT OF SURGICAL ANÆSTHESIA ON THE REACTION OF THE BLOOD.

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It has been shown by Leathes (1), Collip and Backus (2) and others that variations in respiratory activity or in the rate of lung ventilation are reflected at once in the partition of acid and basic phosphates secreted by the kidney, and to a less extent in the rate of elimination of urinary ammonia. Thus Leathes (1) has attributed the morning alkaline tide in the urine to increased respiratory activity on waking, and it would similarly follow that the high degree of acidity of the night urine is due to a lessened degree of lung ventilation during the hours of sleep. Leathes (1) has shown that the carbon dioxide content of the alveolar air is higher during sleep than it is during the waking hours, while Collip (3) has demonstrated that there is no increase, and in some instances that there is an actual decrease, in the bicarbonate content of the blood of the sleeping individual. This points to an actual decrease in the C_H of the blood during sleep. This latter phenomenon must therefore be considered in the explanation of the highly acid urine secreted during sleep.

It has been assumed, since surgical anæsthesia causes a decrease in the alkali reserve of the blood, that the induction of anæsthesia tends toward a condition of acidosis. As has been shown by the theoretical considerations of L. J. Henderson (4) and the experimental work of Hasselbalch (5), Haggard and Henderson (6), Collip and Backus (2), Grant and Goldman (7), and others, a low level of plasma bicarbonate is in itself no indication of an increased C_H of the blood. It is therefore apparent that the C_H of the blood during anæsthesia, or in fact at any time, can only be determined when the ratio of L. J. Henderson () , $\frac{CO_2 \text{ dissolved}}{CO_2 \text{ combined}}$, is known. Owing to the difficulties in the way of obtaining a reliable sample of alveolar air from an anæsthetised person who is undergoing a major surgical operation, it is very difficult to decide whether the C_H of the blood is altered while a patient is under an anæsthetic.

As the observations of Henderson, Haggard and Coburn (6) have demonstrated the beneficial effects of the inhalation of 6 or 8 per cent. of carbon dioxide in the air by patients following major surgical operations, it might be expected that the effect of anæsthesia is not to raise the C_H of the blood, but rather to lower it.

Baldwin (9) has pointed out that the urine becomes more acid during anæsthesia. Reimann and Bloom (10) found that the total acetone bodies of the blood increased during anæsthesia, while Reimann and Hartman (11) found that the total acidity of the urine was increased following anæsthesia. They concluded that small but very definite changes in metabolism take place during anæsthesia, and that the reduction of blood bicarbonate which occurs as a result of anæsthesia is due to these changes and not to over-ventilation, as suggested by Henderson and Haggard (12).

In order to determine if the partition of the acid and basic phosphates of the urine secreted during anæsthesia would throw any light on this question a series of unselected cases were studied.

The operations were all performed late in the morning. The patient was instructed to empty the bladder at eight o'clock on the morning of the operation. The urine passed just previous to anæsthetisation was collected and the time noted. As soon as the patient was returned to the ward after operation the bladder was catheterised, the time noted, and the sample preserved. A third specimen of the urine was collected as soon as the patient expressed the desire to urinate. The specimens were shaken with a few drops of chloroform as soon as collected and then carried to the laboratory for analysis. The total volume of each sample was noted, and the rate of secretion for each period calculated in terms of cubic centimetres secreted per hour. Analyses were made on 10 c.c. of the various specimens. The relative acidity and the concentration of phosphates were determined by the method of double titration, using methyl orange and phenolphthalein as indicators, as outlined by Leathes (1), and later used by Collip and Backus (2). The acidity of the specimen determined in this manner denotes the ratio of acid to basic phosphate. Thus an acidity of 70 per cent. means that 70 per cent. of the total phosphates are present as acid salt, while 30 per cent. are present as basic salt. Ammonia was estimated by the formol titration method. The results are expressed in tabular form in Table I. The rate of secretion of urine before, during and after anæsthesia and the rate of secretion and the concentration of ammonia and phosphates for like periods are herein expressed.

The average duration of anæsthesia was one hour. From fifteen to thirty minutes must be added to this to give the time of secretion of the second specimen. This was due to the delay necessarily ensuing in moving the patient from the ward to the operating theatre and back again. The anæsthetics used were in all instances but one ethyl chloride and ether. Nitrous oxide and oxygen was used in this one instance.

The results show that there is considerable variation in the various factors determined in the different cases. Out of fifteen cases, nine showed a slight increase in the rate of secretion of urine during anæsthesia over that manifested immediately prior to the induction of anæsthesia; one showed no change, while in five the rate was decreased. The acidity of urine or the ratio of acid phosphate to total phosphate was increased in fourteen out of fifteen cases, while in one case this factor was decreased very slightly. The rate of secretion of ammonia was increased to a slight amount in nine cases and diminished in six cases. The rate of phosphate elimination was just noticeably decreased in ten cases and increased in five cases. Results such as these would indicate little or no alteration in the reaction of the blood during anæsthesia. They do not in the least suggest a condition of alkalosis.

There is undoubtedly, in most major surgical operations, increased lung ventilation, which would naturally lead to the manifestation of typical symptoms of temporary alkalosis, such as the disappearance of alkali from the blood by way of the tissues and the urine, unless such reactions were counteracted by some other factor. It is possible that two processes are involved which are mutually antagonistic, and that in the majority of cases they more or less counteract one another in such a way that no appreciable change in blood reaction is brought about. These processes, which are mutually antagonistic, might be termed "the acapnial process" and the "acidotic process of Haggard and Henderson."(13) Increased lung ventilation during anæsthesia would tend to lower the bicarbonate content of the blood, while metabolic disturbances due directly to the anæsthetic would, by the production of acid substances, also tend to lower blood bicarbonate. These two processes when met with separately are, as Haggard and Henderson (13) have pointed out, the direct opposite of each other, although their effect on the blood bircarbonate is, from a superficial view-point, the same. When, however, they are met with simultaneously in the same individual, the one process must necessarily combat or neutralise the other.

To put this theory to the test: a dog was etherised and a cannula placed in the left ureter. A tracheal cannula was then inserted and the anæsthetic given by the Woulff bottle method. The animal was undisturbed for two hours. The urine which had been secreted during this time was then titrated as described above, and found to have an acidity of 50 per cent. Violent hyperpnœa was then induced by intermittent faradic stimulation of the sciatic nerve. This was continued for two hours. The urine secreted during the period of hyperpnœa was then titrated and found to have an acidity of 40 per cent.

Attempts were also made to determine the carbon dioxide content of the alveolar air of dogs during anæsthesia at the same time that the concentration of blood bicarbonate was estimated by the method of Van Slyke and Cullen.(14) The alveolar carbon dioxide tension was determined approximately by a modification of the Plesch (15) method. The animal was allowed to breathe for thirty seconds into a rubber football bladder containing 600 c.c. of expired air. The air in the bladder was then passed through the

Table Showing the Effect of Anæsthesia upon the Urine.

Subject.	Rate of secretion of urine per hour before.	Ditto—during.	Ditto—after.	Acidity of urine before.	Ditto—during.	Ditto—after.	Rate of NH_3 secretion (mgrm. per hr.) before.	Ditto—during.	Ditto—after.	Concentration of NH_3 (mgrm. per 100 c.c.) before.	Ditto—during.	Ditto—after.	Rate of phosphate* excretion before.	Ditto—during.	Ditto—after.	Concentration of phosphate* before.	Ditto—during.	Ditto—after.	Remarks.
1	90	35	30	10	67	68	13·5	12	12	15	35	40	27	23	17	30	66	56	Gas-oxygen anæsthesia.
2	60	20	30	70	74	70	30	10	18	50	50	60	21	7·6	12·6	35	38	42	Abdominal scar removed.
3	40	15	25	70	65	67	12	4	8	30	25	34	14	5·7	10·5	35	38	42	Appendectomy.
4	20	20	45	68	70	80	26	18	54	130	90	120	25	20	67·5	125	100	150	Nerve-graft.
5	55	15	15	75	80	80	70	28·5	28·5	140	190	190	55	16·6	30	100	111	200	Hernia.
6	55	20	30	30	80	76	16·5	19	25·5	30	95	85	33	24	42	60	120	140	Varicocele.
7	30	15	35	60	84	80	31·5	13·5	40	105	90	115	21	12	42	70	80	120	Arthrodesis.
8	40	60	20	10	15	60	8	10	14	20	17	70	16	24	20	40	40	100	Hæmorrhoids.
9	25	35	30	50	60	70	10	10·5	10·5	40	30	35	12·5	14	24	50	40	80	Appendectomy.
10	20	35	10	55	70	70	24	30	13	120	85	130	26	39	16	130	111	160	Hæmorrhoids and septum.
11	35	85	—	38	48	—	24	72	—	68	85	—	30	80	—	85	94	—	Inguinal hernia.
12	40	45	—	54	74	—	32	33	—	80	94	—	28	22	—	70	50	—	Varicose veins.
13	55	30	—	8	77	—	14	18	—	26	60	—	14·3	15·6	—	26	52	—	—
14	40	35	—	58	74	—	29	21	—	73	60	—	20	13·3	—	50	38	—	—
15	50	25	—	50	67	—	27·5	32	—	55	130	—	25	19	—	50	75	—	Bone graft.
16	—	75	20	—	80	74	—	22·5	12	—	30	60	—	22·5	11·4	—	30	57	Cranial defect.

* Phosphate is expressed in terms of c.cs. of $\frac{N}{10}$ acid required to carry 100 c.c. of urine from the phenolphthalein to the methyl orange point.

apparatus of Fridericia and the percentage of carbon dioxide determined. The method is not entirely satisfactory, but the results are nevertheless suggestive. A protocol of such an experiment follows:

Dog, ♂, 16 kilo.

2.25 p.m.	Under ether.				
2.30 ,,	Blood-plasma from femoral vein $CO_2 = 42·4$.				
2.40 ,,	Ureters cannularised.				
2.50 ,,	Tracheal cannula inserted.				
3.00 ,,	Alveolar air	$CO_2 = 5·2$	per cent.		
3.00 ,,	Blood-plasma	$CO_2 = 35$	,,	Ratio $\frac{CO_2 \text{ dissolved}}{CO_2 \text{ combined}}$	= 0·0852.
3·05 ,,	Alveolar air	$CO_2 = 5·2$	,,		
4.05 ,,	Left kidney secreting.				
4.30 ,,	Alveolar air	$CO_2 = 4·0$	,,		
4.30 ,,	Blood-plasma	$CO_2 = 28$	,,	Ditto.	= 0·0819.
5.30 ,,	Alveolar air	$CO_2 = 4·85$	,,		
5.30 ,,	Blood-plasma	$CO_2 = 28$	,,	,,	= 0·0841.
6.00 ,,	Alveolar air	$CO_2 = 4·25$	,,		
6.00 ,,	Blood-plasma	$CO_2 = 28$	,,	,,	= 0·0834.
8.30 ,,	Alveolar air	$CO_2 = 4·25$	,,		
8.30 ,,	Blood-plasma	$CO_2 = 28$	,,	,,	= 0·0834.

Urine secreted 70 per cent. acid. 130 mgrm. NH_3 per 100 c.c.

The ratio $\frac{CO_2 \text{ dissolved}}{CO_2 \text{ combined}}$ calculated for different periods in the experiment would in each case indicate an increased C_H of the blood. It is of interest, however, to note there was slight fluctuation in this factor at different periods. The fact that it was higher in the first instance than in any other would suggest that increased lung ventilation was counteracting the acidotic process due to anæsthesia.

As stated above this method is not beyond criticism, but the further application of it may furnish useful information.

SUMMARY.

(1) The rate of elimination of water, acid, and basic phosphates and ammonia by the kidney in periods before, during and after anæsthesia has been determined in a number of cases.

(2) The acidity of the urine, as estimated by this method, is not materially altered during surgical anæsthesia.

(3) A decrease in the C_H of the blood during surgical anæsthesia is not indicated.

(4) The acidity of the urine may be decreased during anæsthesia by the induction of hyperpnœa.

It is suggested that during surgical anæsthesia two processes, which are mutually antagonistic, are active, one the "acidotic process" of Haggard and Henderson due directly to disturbance in metabolism as a result of anæsthesia, the other the over-ventilation process.

Whether the C_H of the blood falls, rises or remains unchanged during surgical anæsthesia would therefore depend upon the relative intensities of these two processes.

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THE INFLUENCE OF THE REACTION OF MEDIA AND OF THE PRESENCE OF BUFFER SALTS ON THE METABOLISM OF BACTERIA.

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The physical chemical ideas of neutrality, and the character of so-called buffer salts have been associated for some considerable time with physiological problems involving the chemistry of respiration and its connection with the behaviour of blood. It is only a fairly recent development that the bacteriological conceptions of alkalinity and acidity have been diverted from results obtained by titration with an indicator to a so-called "neutral point," to those where the neutral point has been that given by the hydrogen electrode or by indicators, the changing-point of whose range is definitely known.

The influence of buffer substances on growth has also been imperfectly understood. The content of these substances varies enormously in different media used by the bacteriologist, and may range from a practically negligible quantity in the synthetic media used by Galimard, Winogradsky and others to strong solutions of peptones or meat media, where a very considerable amount of acid may be produced in the medium by the metabolism of bacteria without appreciably changing the true reaction.

It will not be necessary to go into the theoretical considerations involved in the use of buffer salts, for these have been fully reviewed in previous work by Henderson, whose paper with Webster, published some twelve years ago, is one of the first real contributions concerning the influence of the reaction on growth.

More recently Clark and his co-workers, Cole and Onslow, Wyeth and Svanberg, have furnished us with important data concerning the influence of the reaction of the medium on the growth of bacteria, so that bacteriologists are now commencing to replace the older notions of alkalinity with those which have a physico-chemical basis.

The present set of experiments was undertaken with a practical object which was of considerable urgency during the war.

In the preparation of toxins for immunisation, considerable difficulty was experienced in obtaining a product of high titre. The batches of toxins varied in toxicity and were stable for a comparatively short time, and then only in the cold. Certain information derived from other members of the Anaërobe Committee of the Medical Research Council indicated that the initial reaction of the medium played an important part in the production of a potent toxin. It seemed also that the "fragility" of the toxin on keeping might be connected with the final reaction of the medium, and that the qualities of added buffer salts might be profitably employed to protect the medium from a too acid final reaction.

It is also possible that in the preparation of bacterial emulsions for vaccines the reaction of the medium might have an influence on the immunising quality of the bacteria themselves. This phase of vaccine production has not, so far as I am aware, been subjected to critical investigation.

As Harris has shown, a marked constancy in metabolism can be obtained when an organism is allowed to ferment in a medium of constant composition. It was therefore determined to compare the effect of bacteria fermenting in the same medium with and without a buffer salt. An opportunity was taken of comparing the action of various buffer salts on bacterial metabolism. We know from the work of Sörensen, to whom we owe the real initial impetus to this type of investigation, that the range in which the buffering by salts takes place is an individual quality, so that it would be obviously useless to employ a salt whose buffer range was on the acid side of any supposed critical point in fragility.

On the other hand, it might be useful to start a fermentation at a point considerably more alkaline than the buffer range, and to allow the fermentation to come to the range in which the buffer acted and be kept there for as long a time as possible.

Certain difficulties are encountered in doing this. Some bacteria will not tolerate more than a certain concentration of salts, such as sodium phosphate, and if the amount present be increased above an amount which seems to be pretty narrowly defined, growth is totally inhibited. In any attempt to prevent the reaction of a medium becoming too acid, one is therefore confronted, on the one hand, by the toxicity of the buffer salt, and on the other by the fact that, with a low concentration of these salts, the medium is rendered rapidly acid through the products of metabolism.

EXPERIMENTAL DATA.

As a standard medium the "tryptic broth" digest of Cole and Onslow was employed in all these experiments. This medium has a very constant amino-acid and ammonia content, and for that reason can be safely employed as a substrate.

Only one sugar, glucose, was used. The experiments ought to be repeated with other sugars, because one cannot safely undertake to apply the results obtained with a mono-saccharide to fermentations in which a di-saccharide is the carbohydrate used.

As buffer salts, three were employed. Of these, sodium phosphate, used in the form of the partially dehydrated di-hydrate, occupied the middle place, buffering strongly between P_H 6 and P_H 7·5. On the acid side of this potassium phthalate was used. This absorbs hydrogen-ions with the production of a flat curve between P_H 6 and P_H 4·5. Sodium pyrophosphate was used in the alkaline region of P_H 8. This salt, which, so far as I am aware, has not hitherto been used for this purpose, deserves some attention from bacteriologists who desire a buffered medium with an initial reaction of about P_H 8.

This salt has a formula of $Na_4P_2O_7$, and may change by the action of acids to either the orthophosphate or to the half saturated acid sodium pyrophosphate $Na_2H_2P_2O_7$. It is probably in the change from the tetra-sodium salt to the di-sodium di-hydrogen salt that the buffer action of the compound takes place.

The technique used was essentially that described by Harris in his paper on the comparison of the metabolism of *B. sporogenes* and the "Reading" bacillus.

Two hundred c.c. of medium were employed from which adequate quantities of gas could be secured for analysis. The fixed acids were determined by extracting 100 c.c. of the medium in a continuous ether extraction apparatus of my own design, but which is in many respects similar to the independently constructed apparatus of Koolmann. The extractions were usually carried out for about eighteen hours. After extraction, the ether was evaporated off, the residue taken up in about 500 c.c. of water, and boiled over a free flame until reduced in bulk to about 50 or 75 c.c. In this way practically all the volatile acids are driven off. There may be a slight loss of fixed acids (Harden, Wolf), but this was neglected. The acid solution is treated with an excess of standard alkali and titrated back with acid, using phenolphthalein as an indicator. The process for the determination of volatile acids has been previously described.

PRELIMINARY EXPERIMENT.

The experiments described were not done in the order in which they are presented. The first organism used was *Vibrion septique*. As the practical urgency of the problem was lessened some time after the commencement of the experiments *B. coli* was selected, as owing to the work of Harden, Theobald Smith, Keyes and Gillespie and Clark, much more is generally known about the fermentative characteristics of this bacillus. The experiments with *B. coli* are therefore presented first, and those with *B. sporogenes*, *Vibrion septique* and *B. welchii* follow.

As, however, the first experiments were of an urgent nature, and the results were suggestive, one of these is given in detail.* It affords some information regarding time relationships not found in later experiments.

The idea was that part of the falling off in toxicity of the toxin of *Vibrion septique* was due to the increasing acidity of the medium as the result of the metabolism of the organism. In order to prevent this in a more effective way than by adding calcium carbonate, a certain quantity of the di-sodium hydrogen phosphate was added to the medium. This buffers strongly in the region of P_H 7·2. The salt used was the di-hydrate. The amount of phosphate added is not without consequence. When 5 per cent. of the salt is added to a peptone bouillon containing 0·2 per cent. of glucose and the medium is inoculated with *V. septique*, fermentation is considerably delayed. The resulting toxin is less potent than when smaller quantities of the buffer are used. Metabolism does take place, for considerable quantities of acids are formed. The reaction of the medium changes only slightly. Experiments showed that *V. septique*, when grown in peptone bouillon to which 0·2 per cent. glucose had been added, produces volatile acids equivalent to about 20 c.c. of a decinormal acid solution per 100 c.c. of medium. If 20 c.c. of decinormal hydrochloric acid be added to peptone bouillon containing 2 per cent. of the di-hydrated di-sodium phosphate, the reaction changes from P_H 7·1 to P_H 6·91. Hence this concentration of the salt should be ample to buffer the medium. That this is the case the following experiment will show :

A stock medium was prepared, consisting of—

Casein digest (tryptic broth)	333 c.c.
Double strength Lemco extract	270 ,,
Sodium chloride 0·5 per cent.	297 ,,

This medium was adjusted to a reaction of P_H 7·3. Fifteen hundred c.c. of this medium were placed in Winchester quart bottles and arranged for fermentation purposes as described by Wolf and Harris.

To one of the bottles 30 grm. of sodium phosphate were added ; the other was left without the buffer salt. After sterilisation a quantity of sterile glucose solution was added, sufficient to make the contents of each flask equivalent to 0·2 per cent. of the sugar.

For inoculation the "Stokes" strain of *V. septique* was used, 10 c.c. from a cooked meat culture being used as the inoculum in each case. The organism had been prepared by animal passage through a guinea-pig, the muscle being planted in peptone bouillon, and a single plating made from the culture. A single colony was picked off and planted into cooked meat, which culture was used directly for inoculating the experimental culture. At intervals readings of the gas production were made, samples taken for analysis, and experiments performed to determine the toxicity.

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* In the earlier experiments I was assisted by Mr. I. E. J. Harris, B.A., B.Sc., to whom it is a great pleasure to acknowledge my thanks.

An examination of the table reveals that while the production of gas is much less in the phosphated medium, the amounts of ammonia and volatile acids in the two fermentations are practically the same. The constancy of the reaction of the buffered medium is continued longer in the buffered than in the unbuffered medium. The curve of ascent of acidity is less steep.

The most notable difference is in the potency of the two toxins so produced. Samples were taken at 24 hours, 48 hours and 96 hours. These were centrifuged at high speed, and the supernatant liquid injected into the marginal ear veins of rabbits.

The results may be tabulated as follows:

Plain Medium.

Hours.	Dose.	Weight (lbs.).	Death.	Time.
24	0·5	2·25	+	18 hours
48	0·5	2·25	+	1 hour
72	0·25	?	0	—

Phosphated Medium.

Hours.	Dose.	Weight (lbs.).	Death.	Time.
24	0·5	2·25	+	18 hours
48	(*a*) 0·5	2·25	+	6 mins.
	(*b*) 0·5	6	+	6 ,,
72	(*a*) 0·25	3·5	+	2 ,,
	(*b*) 0·125	4·5	—	—
96	(*a*) 0·25	4	+	12 ,,
	(*b*) 0·125	4·25	—	—
240	0·25	5	+	10 ,,
528	0·5	6	—	—

One c.c. of a 5 per cent. phosphate bouillon solution when injected into rabbits caused no inconvenience.

It will thus be seen, from the above experiments, that it was possible to retain the toxicity of a peptone meat-extract toxin of *V. septique* at 37° C. for ten days with a high potency till the end of this period. Miss Robertson has been able to prepare a highly toxic product by inoculating plain broth or glucose broth medium which has been adjusted to a reaction of P_{H} 7·8–8 with bits of infected liver of guinea-pigs dead of *V. septique* infection. It is possible that this method allows the formation of toxin, and enough time to elapse before the medium becomes sufficiently acid to permit a toxin destruction.

The analytical results shown in the following table are instructive. They show that metabolism has proceeded in the buffered and unbuffered media very much to the same extent, except in two characteristics. One of these is the gas formation; the other is the true reaction.

In the case of the "plain" medium there was an abundant evolution of gas, amounting to 448 c.c. in 24 hours. Less than one quarter of this volume of gas was evolved by the phosphated medium. In the same length of time the "plain" medium had reached its highest acidity, while the phosphate culture had only increased from $P_H = 7·3$ to $P_H = 6·84$. It will, however, be noticed that the total amount of gas from the phosphate medium when the experiment was continued for 144 hours was about four-tenths of that yielded by the unbuffered medium. The final value at the end of 96 hours was only $P_H = 6·68$. It is worthy of especial note in this connection that the volatile acid production in the two media was identical.

No. of hours.	Gas: c.c. per litre.	Total NH_2N: mgrm. per 100 c.c.	NH_2N formed: mgrm. per 100 c.c.	Total NH_3N: mgrm. per 100 c.c.	NH_3N formed: mgrm. per 100 c.c.	Reaction P_H.	Total volatile acids: c.c. N/10 sol. per 100 c.c.	Volatile acids formed: c.c. N/10 sol. per 100 c.c.
0·2 *per cent. Glucose-peptone-bouillon.*								
0	0	94	0	24	0	6·9	6	0
24	448	95	1	29	5	5·45	21	15
48	533	—	—	34	10	5·76	21	15
72	574	—	—	34	10	5·82	21·6	15·6
96	595	—	—	36	12	5·82	23·6	17·6
144	604	—	—	36	12	5·87	—	—
240	654	—	—	37	13	5·92	27	21
0·2 *per cent. Glucose-peptone-bouillon plus* 2 *per cent.* Na_2HPO_4.								
0	0	89	0	22	0	7·3	6	0
24	106	93	4	28	6	6·84	19	13
48	174	—	—	32	10	6·77	22	16
72	193	—	—	34	12	6·75	22	16
96	203	—	—	34	12	6·68	22	16
144	211	—	—	—	—	—	—	—
240	—	—	—	36	14	6·67	31·6	25·6
528	—	—	—	—	—	6·8	—	—

The main points which will be noticed in the effect of phosphates on the general character of the metabolism of *V. septique* are the following: There is at the end of 144 hours nearly three times as much gas formed by the non-buffered medium as in that to which phosphate has been added. The features of the nitrogen metabolism, as represented by the ammonia production, are substantially the same in the two media, and the amount of volatile acids is alike. In the reaction, however, there is a marked difference.

While in the unbuffered medium at the end of 24 hours one has reached the highest acidity represented by $P_H = 5·45$, in the buffered medium the reaction has become more acid only by a change represented by a rise of hydrogen-ion concentration from $P_H = 7·3$, the initial reaction, to $P_H = 6·84$. Allowing the bacteria to ferment for a further 216 hours produced a subsequent rise to only P_H 6·67. Doubling the time caused what seems to have been a slight reversion, the hydrogen-ion concentration falling to P_H 6·8 at the end of 528 hours. The main features, therefore, of this experiment are that gas production is depressed by the addition of phosphates, while ammonia formation and volatile acid formation are not greatly influenced. The addition of the buffers has a decided influence on the final reaction of the medium, preventing the increase in acidity of the final product.

Reilly, Hichinbottom, Henley and Thaysen describe the influence of calcium carbonate on the production of acetone from maize mash. Here the attempt to keep the medium in the neighbourhood of neutrality is accompanied by the almost complete disappearance of *n*-butyl alcohol and acetone from the final product, while the formation of acetic and butyric acids was greatly increased.

Obviously the process in which phosphates maintain the medium in the neighbourhood of the neutral point is quite a different one from that observed by Reilly and his co-workers when using calcium carbonate.

EXPERIMENTS WITH *B. COLI.*

The organism used in these experiments was the H strain employed by Wyeth in this laboratory in his study of the effects of acids on the growth of this organism.

A preliminary test was made to ascertain the amount of phosphate which would be necessary to inhibit fermentation in plain peptone, and in a peptone medium to which glucose to a concentration of 2 per cent. had been added. The highest concentration of Na_2HPO_4 expressed as the anhydrous salt was 3 per cent.

The results were as follows:

Per cent. Na_2HPO_4.	Plain.		Glucose.	
	Gas in 6 hrs.	P_H 23 hrs.	Gas in 6 hours.	P_H 23 hours.
3	?	7·7	+	6.5
2	+	7·5	++	6·1
1·5	++	7·5	++	5·6
1	++	7·3	++	5·2
0·5	++	7·2	+++	5·2

Gas was formed in 3 per cent. phosphate in plain peptone in 23 hours, but there was a distinct slowing of the gas evolution, and it was decided on the strength of this experiment not to exceed a concentration of sodium phosphate of 2 per cent.

The series of experiments performed with *B. coli* were as follows:

(1) The effect of sodium phosphate on media containing varying concentrations of glucose.

(2) Following these were experiments in which a salt, sodium pyrophosphate, buffering in an alkaline region was used.

(3) Experiments with a salt, acid potassium phthlate, buffering in an acid region.

The experiments will be discussed together, and the individual points may be seen by a reference to the tables printed at the end of the paper.

With *B. coli* the experiments include media containing 0·5 per cent. glucose (Experiments B and F), and media containing 1 per cent. glucose (Experiments D and H). The concentration of sodium phosphate was in each case 2 per cent. reckoned as $Na_2HPO_4 . 2H_20$. As controls, similar media containing the same concentration of carbohydrate, but without the buffer salt, were fermented at the same time and under the same conditions of inoculum and temperature. These controls are fermentations A, C, E and G. A and C, and E and G are the same media, as will be seen from the analytical results.

As the glucose was added after sterilisation and the phosphate had but little effect on the composition of the nitrogen constituents of the media, the nitrogen partition of the buffered medium may be taken as that of the unbuffered.

Sodium Phosphate Experiments.

An examination of the gas evolution induced by *B. coli* reveals a similar situation to that seen with *V. septique.*

In Experiments A and B, in which a concentration of 0·5 per cent. glucose was used, the addition of phosphate retarded gas formation ; at the fifth hour only 6 c.c. had been evolved in the buffered medium, while 91 c.c. were obtained from the plain solution. At the tenth hour 197 c.c. and 81 c.c. were obtained respectively. The final readings, taken about the twenty-ninth hour, showed a difference between 245 c.c. and 178 c.c.

This difference was more pronounced when the concentration of glucose was increased to 1 per cent. Very little more gas was produced from the phosphated medium than when 0·5 per cent. of sugar was employed, while in the unbuffered solution the effect of increasing the amount of sugar was plainly seen in increased gas production. This difference continued up to the eleventh hour. At this point the phosphate medium commenced to increase its gas production, and at the twenty-sixth hour the two media had produced gas within 13 c.c. of one another.

In Experiments E and F, where fermentation was continued for 48 hours with a 0·5 per cent. solution of glucose, one finds that the increasing time allows the phosphated solution to produce nearly the same amount of gas as the medium without the salt.

The results of Experiments G and H are interesting and instructive. Here 1 per cent. glucose was employed and the fermentation was continued for 36 hours. Up to the sixth hour the production of gas in the phosphate medium had lagged behind. From that time on it gathered speed. At 24 hours the plain medium had produced its maximum amount of gas, 284 c.c., while at that time 375 c.c. were produced in the phosphated medium. This amount was further increased to 387 c.c. at the thirty-sixth hour.

It seems that the phosphates mainly influence gas production in its time relation. A very well-marked lag is effected. In two media of similar composition, while the earlier quantities of gas are smaller when phosphate is employed, the total quantity may be considerably larger if the fermentation is prolonged. The reason for this is that fermentation is inhibited early in the plain medium by the production of acid and gas production ceases.

In the phosphated solution protection is afforded from increasing hydrogen-ion concentration, and consequently gas production continues for a longer time and in increasing quantity.

The effect of phosphates on the composition of the evolved gases in this series of experiments is quite uniform. In all cases relatively more hydrogen is evolved when phosphates are present in the medium. In connection with this, as one might expect, there is an increased production of both volatile and fixed acids in the phosphate medium, except in one instance (Experiments E and F), where the fixed acid production is lower in the phosphated medium. There was insufficient material to repeat these analyses.

It seems clear, however, that phosphates influence the carbohydrate metabolism of *B. coli* in increasing gas and acid production, and to a certain extent in modifying the qualitative character of the reaction. An examination of the volatile acid products, as was done in the case of *B. welchii* by Wolf and Telfer, would throw some light on this matter.

With regard to the effect of phosphates on the nitrogen metabolism, although one has noted a very obvious difference in the toxicity of buffered and non-buffered *V. septique* cultures, one does not find any indication in the coarse processes as studied from ammonia and amino-acid determinations. It would seem that rather more ammonia is produced, but the differences in the amino-acid analyses are such that it would be difficult to draw any plausible conclusions.

The effect of adding phosphates on the final reaction of the medium is shown in experiments C and D. Where the final reaction in a 1 per cent. glucose peptone medium rose to P_H 4·68 in 26 hours, the reaction of the the medium to which phosphate had been added rose only to P_H 6·3, although nearly twice as much volatile acids had been formed from the glucose.

The Influence of Sodium Pyrophosphate on the Metabolism of B. coli.

As has been mentioned, Miss Robertson and others have found increased potency of the toxins prepared when the starting-point of the medium was more alkaline than P_H 8. The usual mixtures used in preparing buffer solutions in this region are those containing borates, which are unsuitable for introducing into media. It seems that sodium pyrophosphate might be suitable for this purpose. Unfortunately no titration curves have been made with this salt, but a 2 per cent. concentration of this salt will produce a reaction of $P_H + 8$. Preliminary experiments showed that it was not toxic in concentrations of this amount. As we already had sufficient data regarding the unbuffered media, two experiments were performed in which the concentrations of sugar were 1 per cent. and 2 per cent. respectively.

The composition of the gas varies considerably between the two experiments, the higher concentration of carbon dioxide occurring with the higher concentration of glucose.

The final reactions of the two experiments show that 2 per cent. of sodium pyrophosphate is insufficient to buffer the amount of volatile and fixed acids produced in a 2 per cent. glucose medium. The amount of volatile and especially of fixed acids is very much greater than was observed in any of the other experiments. In the 2 per cent. glucose fermentation, the hydrogen-ion concentration had risen to P_H 4·80, which is in the neighbourhood of the acid inhibiting point. Although volatile acids corresponding to 40·2 c.c. of N/10 alkali, and fixed acids corresponding to 45·6 c.c. of N/10 alkali were produced in the 1 per cent. glucose solution, the hydrogen-ion concentration had only risen to P_H 6·08 from P_H 8·24. It would appear, therefore, that sodium pyrophosphate may serve a very useful purpose where a buffer salt is required for bacteriological media when a low hydrogen-ion concentration is desired.

The Effect of Potassium Phthalate on the Metabolism of B. coli.

The "reaction resultants" of *B. coli* in media containing phthalates have been studied in detail in my laboratory by Wyeth, and this salt had previously been used by Clark in his study of *B. coli*. No work has been done on the metabolism of organisms grown in a medium containing the salt. It was realised at the outset that its buffer qualities were confined to a rather high hydrogen-ion concentration, and that the drop from an initial hydrogen-ion concentration of $P_H \pm 7·5$ would be sudden, as no buffering takes place at this point. The fermentation was continued for 47 hours, and the concentrations of glucose were the same as in the preceding experiments, with which it can usefully be compared.

The gas production falls somewhat behind that of the pyrophosphate fermentations, and the relative concentration of carbon dioxide is between the two figures obtained there.

The volatile acid production corresponds with what was found in using phosphates as a buffer, but appears to be slightly lower than what is obtained with sodium pyrophosphate. Owing to the presence of phthalic acid the fixed acid production could not be ascertained.

There is nothing noteworthy in the nitrogen metabolism. The figures for ammonia production and amino-acid consumption are well within the limits found in the preceding experiments.

EXPERIMENTS WITH *B. WELCHII.*

The experiments performed with *B. welchii* are in many respects similar to those done with *B. coli*. It will not be necessary to discuss them in detail, but only the principal points will be noted, especially in so far as they show a difference from the facultative anaërobe. Any special details may be obtained from the tables themselves.

Fermentations were carried out with media containing 2 per cent. of sodium phosphate with 0·5 per cent. glucose (Experiments S and T) and with 1 per cent. glucose (Experiments Q and R). The main point in the experiments with *B. welchii* is the effect of phosphates on gas production. While these salts delayed the evolution of gas by *B. coli*, it was found that when the experiment was continued long enough, nearly the same amount of gas was produced from the phosphated medium. This is not the case with *B. welchii.* Not only is the gas production delayed, but the amount is never so large. In the Experiments Q and R for example, 564 c.c. of gas were evolved from the plain medium in 9 hours, while only 14 c.c. of gas were evolved from the same volume of phosphated medium. In Experiments S and T the plain medium had produced 293 c.c. of gas in 52 hours, while from the phosphated medium only 7 c.c. were obtained. After a fermentation lasting 219 hours 167 c.c. of gas were evolved, while the amount from the plain medium was 314 c.c.

The phosphate had a distinct effect on the composition of the evolved gas, the carbon dioxide content of which rose to 66·2 per cent. It is, however, difficult to see any evidences of a difference in the direction of the nature of the reaction being shifted from an examination of the volatile or fixed acid fermentation. Both these products are formed to the same extent in the plain medium as in the phosphated.

The nitrogen metabolism does not show any changes which will account for the differences in the gas production. In other words, carbohydrate and nitrogen metabolism, in so far as the analytical results go, do not disclose the reason for the very profound difference in gas formation due to the presence of phosphates. A more complete examination of the products of fermentation is required.

EXPERIMENTS WITH *VIBRION SEPTIQUE.*

The experiments recorded here may be discussed shortly, as the results obtained are in practically every respect similar to those obtained in the larger fermentations described at the commencement of the paper. They enlarged these results in so far as beef extract was excluded from the medium, and the concentration of glucose was varied from 0·5 per cent. to 1 per cent.

The most marked feature of these experiments is the suppression of the gas production when the experiment is not too long continued. Thus in Experiments AE and AF, when the fermentation lasted 156 hours, only 7 c.c. of gas were detected in a 0·5 per cent. glucose peptone medium, while the control had reached its maximum in 77 hours with 235 c.c. An even more striking example is furnished by Experiments AG and AH, where 1 per cent. glucose was used. With an evolution of 432 c.c. of gas by the control fermentation in 156 hours, 3 c.c. were evolved by the phosphate medium—an amount which is within the limits of experimental error.

A survey of the analytical results would make it appear that little metabolism was taking place, and this is more especially the case in volatile acid formation. Except in experiments AB, and especially experiment V, there is no evidence of volatile acid formation. (In V there has been gas formed to the extent of 41 c.c.) But in all cases metabolism has taken place as judged by the fixed acids produced. That viable organisms were present in every case was ascertained by sub-inoculations.

It is a matter of practical interest that no potent toxin was ever obtained in any of these experiments. Whether the use of meat extract is absolutely necessary for this purpose must be the subject of future investigation.

EXPERIMENTS WITH *B. SPOROGENES.*

As all the foregoing experiments were carried out with essentially saccharolytic organisms, it was necessary to complete the series by an examination of the effect of buffer salts on two proteolytic organisms. For this purpose *B. sporogenes* (Metchnikoff) and *B. histolyticus* were chosen. These had been the subject of previous study by Harris and myself. The concentrations of glucose employed varied from 0·5 per cent. to 2 per cent.

The effect of phosphates on fermentation by *B. sporogenes* are shown in the lag in gas production in the shorter experiments (AN and AR). In one experiment, AP, where the concentration of glucose was 2 per cent., this lag was not seen, and the amount of gas produced by the phosphated medium was always in excess of that formed by the "plain."

The nitrogen metabolism as gauged by the ammonia and amino-acid production is not altered to any remarkable extent by the introduction of buffer salts into the medium. On the whole neither the amino-acid production nor the ammonia production are quite as high as in the plain medium, but the differences are so small that it is not possible with the number of experiments performed to make any definite statement.

The volatile acid production seems to be slightly depressed, while the fixed acid is increased in quantity by the introduction of phosphates. No decisive change takes place in the composition of the evolved gases.

It will be noted that, as in the previous experiments, the addition of phosphates has influenced the final reaction of the media, the phosphate fermentation showing a lower hydrogen-ion concentration. As the growth of *sporogenes* does not tend to a very acid end-point, the differences between the two are not great.

EXPERIMENTS WITH *B. HISTOLYTICUS*.

The series of experiments concludes with fermentations of *B. histolyticus*—an organism which is essentially proteolytic. The chemical activities were studied by Wolf and Harris (1918), but no fermentations in glucose-peptone were carried out. It only forms very large quantities of gas when fermented in cooked meat, and the composition of the gas is distinguished by being almost completely carbon dioxide. Three experiments were performed with this organism, the concentrations of glucose varying between 0·5 and 2 per cent. It is noticeable that, although the amount of gas evolved is very small, phosphates act in the same direction as with saccharolytic types of organisms. The amount in a phosphate medium is always below that of the plain.

The effect on nitrogen metabolism is distinct. The ammonia formation is high in both media, but the production in the presence of phosphates is always less. In two of the experiments there was a consumption of the amino-acid portion of the medium. In the third (AW and AX) the amount of amino-acids had increased as the result of fermentation.

The acid production was not influenced to any great extent by the presence of a buffer salt; the fixed acids seem to be slightly increased in consequence.

Owing to the amount of ammonia produced in fermentation the final hydrogen-ion concentration is low, but in all cases increase in acidity was inhibited by the presence of phosphates.

SUMMARY.

The effect of moderate concentrations of di-sodium hydrogen phosphate (2 per cent.) on the growth of *B. coli*, *Vibrion septique*, *B. welchii*, *B. sporogenes* and *B. histolyticus* in glucose-peptone has been investigated. The effect of acid potassium phthalate and sodium pyrophosphate on the growth of *B. coli* was also ascertained.

In all cases the buffer salt either partially inhibits gas production or produces a lag. If the fermentation be very prolonged, the volume of gas may finally approach that obtained from a non-buffered medium. The effect of phosphates on gas production is most pronounced in the case of actively saccharolytic organisms, but is also definite with proteolytic bacteria such as *B. sporogenes* and *B. histolyticus*.

The effect of phosphates on the nitrogen metabolism is not pronounced. On the whole the formation of amino-acids and ammonia from a medium containing peptones, polypeptides and amino-acids is not increased.

In the case of the saccharolytic organisms the acid production appears to be increased when buffer salts are added to the medium. This bears out some of the results which have been obtained with similar organisms when fermentation takes place in the presence of calcium carbonate.

The final reaction of the fermentations is affected by the presence of buffer salts, in the sense that in no case did the medium become so acid in the presence of buffer salts as it did when these salts were absent.

The toxin formation in *V. septique* cultures is influenced by the presence of a buffer salt—sodium phosphate. The toxin had a higher potency than the control made without the salt. It is shown that toxins may be kept potent for long periods of time in the presence of sodium phosphate under conditions which would quickly destroy the toxicity of a product made in an unbuffered medium.

TABLES.

TABLE I.—*Experiments A and B.* B. coli *in* 0·5 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	A. Plain.		B. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·412	0·385	0·440	0·407
Ammonia nitrogen: mgrm. per 100 c.c.	22·4	33·3	22·4	39·5
Amino-acid nitrogen: mgrm. per 100 c.c.	138	141	137	118
Volatile acids: c.c. N/10 acid per 100 c.c.	3·8	23·6	5·05	34·6
Fixed acids: c.c. N/10 acid per 100 c.c.	—	36·2	—	37·4
Carbon dioxide per cent.	—	51·3	—	37·4
Hydrogen per cent.	—	48·7	—	62·6
Total gas: c.c. in 28·6 hours	—	245	—	178
P_H 20° C.	7·03	5·36	7·42	6·91

*Gas Production.**

	c.c.	
Hours.	A.	B.
3·4	22	5
5·4	102	10
12	213	100
23·25	237	163
28·4	245	178

TABLE II.—*Experiments C and D.* B. coli *in* 1 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	C. Plain.		D. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·412	0·398	0·440	0·406
Ammonia nitrogen: mgrm. per 100 c.c.	22·4	33·3	22·4	36·2
Amino-acid nitrogen: mgrm. per 100 c.c.	138	112	137	112·5
Volatile acids: c.c. N/10 acid per 100 c.c.	3·8	25·7	5·05	46·3
Fixed acids: c.c. N/10 acid per 100 c.c.	—	29·7	—	?
Carbon dioxide per cent.	—	52·1	—	41·6
Hydrogen per cent.	—	47·9	—	58·4
Total gas: c.c. in 26 hours	—	30·2	—	28·9
P_H 20° C.	7·03	4·68	7·42	6·30

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* In order to save space only a few points are given out of a large number of observations.

Gas Production.

Hours.	c.c. C.	 D.
3·4	35	5
5·4	130	16
12	256	270
23·25	297	283
28·4	302	289

* TABLE V.—*Experiments I and J.* B. coli *in* 1 *per cent. and* 2 *per cent. Glucose-peptone with* 2 *per cent. Sodium Pyrophosphate.*

	I. 1 per cent. glucose.		J. 2 per cent. glucose.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·357	0·332	0·357	0·317
Ammonia nitrogen: mgrm. per 100 c.c.	15	28·1	15	23·4
Amino-acid nitrogen: mgrm. per 100 c.c.	118	98·5	118	89·2
Volatile acids: c.c. N/10 acid per 100 c.c.	4·7	40·2	4·7	32·6
Fixed acids: c.c. N/10 acids per 100 c.c.	—	45·6	—	82
Carbon dioxide per cent.	—	45·9	—	53·2
Hydrogen per cent.	—	54·1	—	46·8
Total gas: c.c. in 49 hours	—	405	—	445
P_H 20° C.	8·24	6·08	8·24	4·80

Gas Production.

Hours.	c.c. 1 per cent. I.	 2 per cent. J.
3	2	1
24	86	422
28·3	244	438
31·15	301	441
49	405	445

TABLE VI.—*Experiments O and P.* B. coli *in* 1 *per cent. and* 2 *per cent. Glucose-peptone with* 2 *per cent. Acid Potassium Phthalate.*

	O. 1 per cent. glucose.		P. 2 per cent. glucose.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·381	0·380	0·381	0·36
Ammonia nitrogen: mgrm. per 100 c.c.	17·8	29·5	17·8	27·2
Amino-acid nitrogen: mgrm. per 100 c.c.	130	110	130	103
Volatile acids: c.c. N/10 acid per 100 c.c.	7·1	27·8	7·1	30·2
Carbon dioxide per cent.	—	51·3	—	51·3
Hydrogen per cent.	—	48·7	—	48·7
Total gas: c.c. in 47 hours	—	406	—	402
P_H 20° C.	+8	5·48	+8	5·25

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* At the request of the Editors, the author has allowed Tables III, IV, XI, XIV and XVII to be omitted.

Gas Production.

	c.c.	
	1 per cent.	2 per cent.
Hours.	O.	P.
7·4	1	91
8·2	2·6	134
8·7	21	166
9·2	39	186
11·2	113	242
23	292	402

TABLE VII.—*Experiments Q and R.* B. welchii *in* 1 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	Q. Plain.		R. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·345	—	0·333	0·308
Ammonia nitrogen: mgrm. per 100 c.c.	19·6	36·4	19·8	34·5
Amino-acid nitrogen: mgrm. per 100 c.c.	114	108	112	111
Volatile acids: c.c. N/10 acid per 100 c.c.	4·2	40·3	6·4	46·7
Fixed acids: c.c. N/10 acid per 100 c.c.	—	14	—	11
Total gas: c.c. in 9 hours	—	564	—	14
P_H 20° C.	7·1	>4·4	7·4	5·9

Gas Production.

	c.c.	
Hours.	Q.	R.
3	55·8	3
5	168	4
7	255	7
9	297	14

TABLE VIII.—*Experiments S and T.* B. welchii *in* 0·5 *per cent. Glucose peptone with and without* 2 *per cent. Sodium Phosphate.*

	S. Plain.		T. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·356	0·398	0·334	0·354
Ammonia nitrogen: mgrm. per 100 c.c.	20·9=	55·4	22·1	41·2
Amino-acid nitrogen: mgrm. per 100 c.c.	128	174·4	116	148
Volatile-acids: c.c. N/10 acids per 100 c.c.	7·7	36·9	16·2	34·3
Fixed acids: c.c. N/10 acids per 100 c.c.	—	31·7	—	28·9
Carbon dioxide per cent.	—	49·7	—	33·8
Hydrogen per cent.	—	50·3	—	66·2
Total gas: c.c. in 219 hours	—	314	—	167
P_H 20° C.	7·01	6·10	7·32	6·8

Gas Production.

Hours.	c.c. S.	c.c. T.
1·25	5	5
8	6	5
22·5	263	6
52	293	7
219	314	167

Table IX.—*Experiments U and V.* V. septique *in* 1 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	U. Plain.		V. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·345	0·315	0·333	0·282
Ammonia nitrogen: mgrm. per 100 c.c.	19·6	22·3	19·8	21·9
Amino-acid nitrogen: mgrm. per 100 c.c.	114	107	112	110
Volatile acids: c.c. N/10 acid per 100 c.c.	4·25	25·9	6·4	33·9
Fixed acids: c.c. N/10 acid per 100 c.c.	—	4·8 ?	—	17·6
Total gas: c.c. in 30 hours	—	138	—	41
P_H 20° C.	7·1	4·8 ?	7·4	6·3

Gas Production.

Hours.	c.c. U.	c.c. V.
3	9	10
5	9	11
8	21	5 ?
24	124	32
30	138	41

Table X.—*Experiments Y and Z.* V. septique *in* 0·5 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	Y. Plain.		Z. Phosphate.	
	Initial.	Final.	Initial.	Final
Total nitrogen per cent.	0·356	0·393	0·334	0·355
Ammonia nitrogen: mgrm. per 100 c.c.	20·9	26·9	22·1	26·9
Amino-acid nitrogen: mgrm. per 100 c.c.	128	137·6	116	134·4
Volatile acids: c.c. N/10 acids per 100 c.c.	7·7	23·9	16·2	30·7
Fixed acids: c.c. N/10 acid per 100 c.c.	—	28·5	—	27·9

	Y. Plain.		Z. Phosphate.	
	Initial.	Final.	Initial.	Final
Carbon dioxide per cent.	—	42·6	—	40·5
Hydrogen per cent.	—	57·4	—	59·5
Total gas: c.c. in 219 hours	—	121	—	105
P_H 20° C.	7·01	5·10	7·32	6·23

Gas Production.

	c.c.	
Hours.	Y.	Z.
1·25	6	8
8	6	9
22·5	23	26
52	50	6?
73	120	55
219	121	105

TABLE XII.—*Experiments AE and AF.* V. septique *in* 0·5 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	AE. Plain.		AF. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·413	0·402	0·442	0·418
Ammonia nitrogen: mgrm. per 100 c.c.	22·5	28·3	4	22·4
Amino-acid nitrogen: mgrm. per 100 c.c.	139·1	143·3	127	141
Volatile acids: c.c. N/10 acid per 100 c.c.	3·8	25·6	5	4·2
Fixed acids: c.c. N/10 acid per 100 c.c.	—	25·1	—	10·4
Carbon dioxide per cent.	—	41·2	—	—
Hydrogen per cent.	—	58·8	—	—
Total gas: c.c. in 156 hours	—	234	—	7
P_H 20° C.	7·01	5·05	7·32	7·32

Gas Production.

	c.c.	
Hours.	AE.	AF.
23	119	4
33·7	160	4
49	205	3
77	235	9
135	234	7
156	234	7

TABLE XIII.—*Experiments AG and AH.* V. septique *in* 1 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	AG. Plain.		AH. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·413	0·401	0·442	0·432
Ammonia nitrogen: mgrm. per 100 c.c.	22·5	28·6	22·4	23·4
Amino-acid nitrogen: mgmr. per 100 c.c.	139·1	141·8	127	132·6
Volatile acids: c.c. N/10 acid per 100 c.c.	3·8	34·1	5	4·2
Fixed acids: c.c. N/10 acid per 100 c.c.	—	26·6	—	9·5
Carbon dioxide per cent.	—	41·6	—	—
Hydrogen per cent.	—	58·4	—	—
Total gas: c.c. in 156 hours	—	432	—	—
P_H 20° C.	7·01	5·17	7·32	7·40

Gas Production.

	Gas c.c.	
Hours.	AG.	AH.
23	233	3
27	253	3
49	370	3
70	415	3
113	431	3
135	430	1
156	432	3

TABLE XV.—*Experiments AO and AP.* B. sporogenes *in* 2 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	AO. Plain.		AP. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·336	0·344	0·368	0·347
Ammonia nitrogen: mgrm. per 100 c.c.	11·2	50	18·4	43·9
Amino-acid nitrogen: mgrm. per 100 c.c.	142	148	131	149
Volatile acids: c.c. N/10 acid per 100 c.c.	4·8	40·2	5·9	48·9
Fixed acids: c.c. N/10 acid per 100 c.c.	—	11·5	—	13·6
Carbon dioxide per cent.	—	64·2	—	60·9
Hydrogen per cent.	—	35·8	—	39·1
Total gas: c.c. in 94 hours	—	415	—	440
P_H 20° C.	7	5·6	7·3	5·8

Gas Production.

Hours.	AO. (c.c.)	AP. (c.c.)
1·5	2·1	4·2
23	153	197
28·5	201	250
47	312	400
71	400	420
94	415	440

TABLE XVI.—*Experiments AQ and AR.* B. sporogenes *in* 1 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	AQ. Plain.		AR. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·356	0·383	0·334	0·366
Ammonia nitrogen: mgrm. per 100 c.c.	20·9	68·7	22·1	65·8
Amino-acid nitrogen: mgrm. per 100 c.c.	128	154	116	138
Volatile acids: c.c. N/10 acid per 100 c.c.	7·7	44·4	16·2	63·3
Carbon dioxide per cent.	—	64·4	—	65
Hydrogen per cent.	—	35·6	—	35
Total gas: c.c. in 78 hours	—	262	—	166
P_H 20°C.	7·01	6·17	7·32	6·44

Gas Production.

Hours.	AQ. (c.c.)	AR. (c.c.)
5	6	8
7	6	8
22	132	19·4
28	179	48·9
47	228	129
78	262	166

TABLE XVIII.—*Experiments AU and AV.* B. histolyticus *in* 2 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	AU. Plain.		AV. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·368	0·363	0·336	0·367
Ammonia nitrogen: mgrm. per 100 c.c.	18·4	75·2	11·2	70·2
Amino-acid nitrogen: mgrm. per 100 c.c.	131	119	142	130
Volatile acids: c.c. N/10 acid per 100 c.c.	5·9	41·8	4·8	50
Fixed acids: c.c. N/10 acid per 100 c.c.	—	3·3	—	9·9
Total gas: c.c. in 94 hours	—	49·5	—	32·6
P_H 20° C.	7·01	6·7	7·32	7

Gas Production.

Hours.	c.c.	
	AU.	AV.
1·5	2·1	1
23	13·7	14·7
28·5	18·9	20
47	24·7	26·8
71	27·4	25·2
94	49·5	32·6

TABLE XIX.—*Experiments AW and AX.* B. histolyticus *in* 1 *per cent. Glucose-peptone with and without* 2 *per cent. Sodium Phosphate.*

	AW. Plain.		AX. Phosphate.	
	Initial.	Final.	Initial.	Final.
Total nitrogen per cent.	0·356	0·394	0·334	0·35
Ammonia nitrogen: mgrm. per 100 c.c.	20·9	84·5	22·1	51
Amino-acid: nitrogen mgrm. per 100 c.c.	128	138	116	126
Volatile acids: c.c. N/10 acid per 100 c.c.	7·7	59·1	16·2	26·6
Total gas: c.c. in 78 hours	—	35·7	—	14·2
P_H 20° C.	7·01	6·38	7·32	7·2

Gas Production.

Hours.	c.c.	
	AW.	AX.
5	1	3·1
7	3·1	4·2
22	10	5·8
28	14·2	5·8
47	25·2	11·5
78	35·7	14·2

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GLYCOGENIC INFILTRATION OF THE LIVER OF LABORATORY RABBITS.

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In March, 1919, I was asked by the Salvarsan Committee of the Medical Research Council to report upon the histological changes in material obtained from rabbits which had been subjected to experiment.

Two groups of experiments, A and B, had been made. In each of these groups one series of rabbits had been inoculated with 0·85 per cent. saline made with contaminated water, whilst another parallel series had been inoculated with salvarsan dissolved in a 0·85 per cent. saline made with the same contaminated water. For convenience I shall employ "W." (water) to represent contaminated saline alone, and "S." to represent salvarsan in contaminated saline. The two types of experiment in each series of the groups A and B can thus be represented by "W. A," "S. A," and "W. B," "S. B," whilst each pair of experiments can be distinguished by the addition of a numeral, *e.g.* "W. A II," "S. A II," "W. B IV," "S. B IV."

The material consisted of microscopic sections of the liver from nineteen experiments, of the kidney from twenty, of the myocardium from seventeen and of the spleen from fifteen. All the tissues enumerated had been embedded in paraffin and had been stained with hæmatoxylin and eosin; there were also a few sections of livers treated with osmic acid, and one frozen section of a liver stained with Sudan III and hæmatoxylin. I was informed that the tissues had been fixed in formaldehyde solution.

HISTOLOGICAL CHANGES.

A comparison of the sections from different cases showed that the most striking differences were exhibited by the parenchyma of the *liver*.

In certain livers there was a very conspicuous vesiculation of the cytoplasm of the hepatic cells, associated with changes in, and absence of, nuclei. I arranged the livers, therefore, on two occasions at a week's interval in series according to the estimated degrees of cytoplasmic vesiculation. On each occasion six groups were separated. The two estimations, however, showed some differences in the order of the individual livers in each group. The subdivision of the groups was, therefore, abandoned.

Group 1.—In W. A IV and S. B III the parenchyma showed no greater vesiculation than is seen in all paraffin sections of the liver. A few nuclei were stained abnormally faintly.

Group 2.—In W. A VI, S. A VII, W. B III, S. B VI and S. B VII the cytoplasm was interrupted by small, clear clefts and spaces. These were frequently perinuclear. In W. A VI and S. A VII the cells in the periphery of the lobules were affected more than those in the centre. In W. B III, S. B VI and S. B VII the central cells were somewhat more affected than the peripheral. Faint staining of nuclear chromatin and absence of nuclei were seen in all; these nuclear changes varied in amount from "slight" to "considerable."

Group 3.—In W. A III, S. A III, and W. B II there was slightly greater vesiculation, and the dissociated cytoplasm was decidedly granular. Faint staining of nuclei was considerable.

Group 4.—In W. A II and S. A II vesiculation was considerably greater, and the cytoplasm was conspicuously granular. In both cases more cells in the centres of the lobules were affected than in the periphery. In both cases faint staining of nuclei was considerable, and was found especially in central cells.

Group 5.—In W. A I vesiculation was much greater, and, in the centres of the lobules, caused a somewhat different appearance. The cells in the central third or more of the lobules were greatly swollen and either round or cubical. These cells were conspicuously vesicular, part of the cytoplasm forming a distinct border, or membrane, to the cell-body, whilst the remainder was scattered in the form of granules, generally united to form a net, throughout a colourless, clear ground. Differences in nuclear structure, and apparent nuclear degeneration indicated by extreme pallor of nuclei and absence of nuclei, were greater than in the previous sections.

Group 6.—In W. B I, W. B IV, W. B VI, S. B I, S. B II and S. B IV the vesiculation was of the type described in the previous paragraph, but was considerably greater. The cells were greatly swollen, and the cytoplasm was almost entirely reduced to a border round the clear cell. The hepatic columns frequently resembled sections of hollow spheres, or hollow cubes, piled in rows, some showing a nucleus, placed centrally, but many failing to do so. Variations in structure, size and in the depth of coloration of the nuclei, and absence of nuclei were greater than in the other livers. The extreme pallor of many nuclei was a conspicuous feature. In W. B IV the maximum degree of vesiculation seldom affected more than the central one-third or two-thirds of the lobules. In W. B I, W. B VI and S. B IV the central two-thirds were chiefly affected. In S. B I the cells in the extreme periphery of the lobules were sometimes free. In S. B II great vesiculation was present throughout the lobules, but was greater in the intermediate and peripheral zones.

The following table gives the estimated degrees of vesiculation of the hepatic cells, expressed by numbers of + signs, in each series of the two groups of experiments A and B.

Table I.

W. A I + + + + +	S. A I (no section).
W. A II + + +	S. A II + + +.
W. A III + +	S. A III + +.
W. A IV −	S. A IV (no section).
W. A VI +	S. A VI (no section).
W. A VII (no section)	S. A VII +.
W. B I + + + + + +	S. B I + + + + + +.
W. B II + +	S. B II + + + + + +.
W. B III +	S. B III −.
W. B IV + + + + + +	S. B IV + + + + + +.
W. B VI + + + + + +	S. B VI +.
W. B VII (no section)	S. B VII +.

The livers also showed various degrees of portal infiltration and fibrosis. These changes were associated with cholangitis. They were exactly similar to the inflammatory changes which accompany coccidiosis. In S. A III and S. A VII there were granulomatous nodules such as are seen in coccidiosis, and in S. A II and S. B VII a few oöcyst capsules of *Eimeria stiedæ*, surrounded by giant-cells, were present within bile-ducts. A table comparing the degree of parenchymatous vesiculation with the degree of portal infiltration and fibrosis showed that there was no trace of correlation between the two conditions. There can be no doubt that the portal infiltration and fibrosis were due to coccidiosis, an infection from which, in my experience, very few laboratory rabbits have been free.

In the *kidneys* there was evidence of parenchymatous degeneration. I made a comparative estimate of the degree of degeneration, employing nuclear degeneration as the criterion for differentiation. I found this to be a matter of great difficulty, there being no such conspicuous differences as were present in the livers. A table comparing the estimated degrees of parenchymatous vesiculation in the livers with the degrees of parenchymatous degeneration in the kidneys showed that there was no correlation between the two conditions.

In six kidneys (S. A III, S. A IV, W. B I, W. B VI, S. B III and S. B VI) there were small focal areas of infiltration. These lay either in the cortex and upper medulla, or in the cortex and intermediate zone, or in the intermediate zone and medulla, or in the intermediate zone alone or in the medulla alone.

Within these areas round and small polygonal, mononuclear cells, occasionally accompanied by spindle-cells, lay in the interstitial tissue, and there was a catarrhal desquamation of the epithelium in the included tubules. The presence of this infiltration bore no relation to the estimated amount of vesiculation of the hepatic parenchyma. On the other hand, these focal inflammatory areas occurred, with one exception, within kidneys in which the degree of parenchymatous degeneration had been estimated to be greatest.

The *myocardium and spleen* did not show any changes of significance.

THE NATURE OF THE CHANGES IN THE HEPATIC PARENCHYMA.

In all the sections of the liver stained with hæmatoxylin and eosin there was a vesiculation of the cytoplasm of the hepatic cells. The livers have been grouped above (p. 311) according to the degrees of this vesiculation.

In Group I the vesiculation was very slight, and, indeed, no greater than is found in all sections of liver embedded in paraffin. In the other groups the vesiculation, judged by morphological characters, which alone can be the test in sections stained with hæmotoxylin and eosin, was similar in kind but showed great variations in degree. The vesiculation was associated with nuclear changes, the estimated frequency and degree of the alterations in the nuclei corresponding closely to the degree of vesiculation of the cells.

Morphologically the vesiculation differed in certain details from that which is seen in paraffin sections when the cytoplasm is affected by fatty infiltration or degeneration. Further, in the few preparations specially stained for the demonstration of fat no fat was shown. Again, I had previously seen a vesiculation, morphologically similar to that in Group 6, in the centres of the hepatic lobules of three rabbits. Two of these rabbits had been given trinitrotoluene. The third was sent to me as a normal control: it was not in reality normal, because there were in the kidneys numerous hyaline and hæmorrhagic casts, and calcification of many cells lining convoluted tubules. In the livers of these three rabbits only very occasional small, fatty granules were demonstrated by Sudan III. It was therefore possible to exclude fatty change as the cause of the vesiculation.

The vesiculation was not that of "hyaline droplet degeneration," because the granules floating in the vacuolar cell-bodies had neither the size, definite spherical shape nor deep eosinophilia of "hyaline droplets."

The lesser grades of the vesiculation and granulation (in Groups 2, 3 and 4) resembled closely in morphology those which express "cloudy swelling" or "albuminous degeneration" in paraffin sections. I considered, therefore, that the great vacuolation seen in Groups 5 and 6 might represent a severe degree of "dropsical degeneration," a condition which I believe to differ from "albuminous degeneration" only in a greater absorption of water by the cytoplasm. The extreme pallor of many of the nuclei and the absence of other nuclei appeared to demonstrate karyolysis, and thus supported the view that the vesiculation was an expression of degeneration.

It appeared strange, however, that such extreme degrees of dropsical degeneration should be present in the liver, and should bear no relation to the degree of parenchymatous degeneration in the corresponding kidneys. Moreover, the changes in the cells in the most severely affected livers reminded me of those seen in cells which I had proved to be loaded with glycogen, for instance in cells lining tubules of the kidney in cases of diabetes and in cells of Grawitz's carcinoma of the kidney. I considered it advisable, therefore, to ascertain the effect of glycogenic infiltration on the liver of the rabbit.

Prof. James McIntosh, at that time working in the department of Prof. William Bulloch, kindly put an apparently normal rabbit at my disposal and assisted me in an experiment. A large bunch of carrots was placed in the rabbit's hutch at ten in the morning. Four hours later the animal was killed instantly by a single blow on the head, and within half a minute of death small portions of the liver had been excised and dropped into absolute alcohol; the alcohol was immediately heated until it boiled. Other portions of the liver were then excised from the margins of the previous excisions, and were placed into 4 per cent. saline formaldehyde solution.

A necropsy on the rabbit revealed to the naked eye no abnormalities except a few necrosed, coccidial areas in the liver and one softened mesenteric gland. Portions of the lungs, kidneys, myocardium, thyroid gland, pancreas, spleen, suprarenal bodies and ovary were fixed in 4 per cent. saline formaldehyde and were embedded in paraffin. Sections were stained with Ehrlich's hæmatoxylin and eosin, Weigert's iron-hæmatoxylin and van Gieson's mixture, and Weigert's fuchselin and neutral red. No abnormalities were detected on microscopic examination.

The portions of liver fixed in boiling alcohol were embedded in celloidin, and sections were stained by Best's carmine method for the demonstration of glycogen. One of the portions fixed in formaldehyde was cut upon the freezing microtome, and sections were stained in Sudan III and hæmatoxylin. The remaining portions were embedded in paraffin, and sections were stained in hæmatoxylin and eosin, etc.

In the sections stained with Sudan III no fat was demonstrated, except in the endothelial cells of Kupffer, in one or two hepatic cells, in the form of a few small granules, and in a few cells of a portal infiltration, which was connected with the coccidiosis.

In the sections stained by Best's method the hepatic cells were loaded with glycogen (Plate-fig. 2).

The appearance of the cells in the material fixed in formaldehyde, embedded in paraffin and stained with hæmatoxylin and eosin is represented in Plate-fig. 1. These sections resembled very closely those of Group 6 above. The majority of the cells most severely affected by the vesiculation lay in

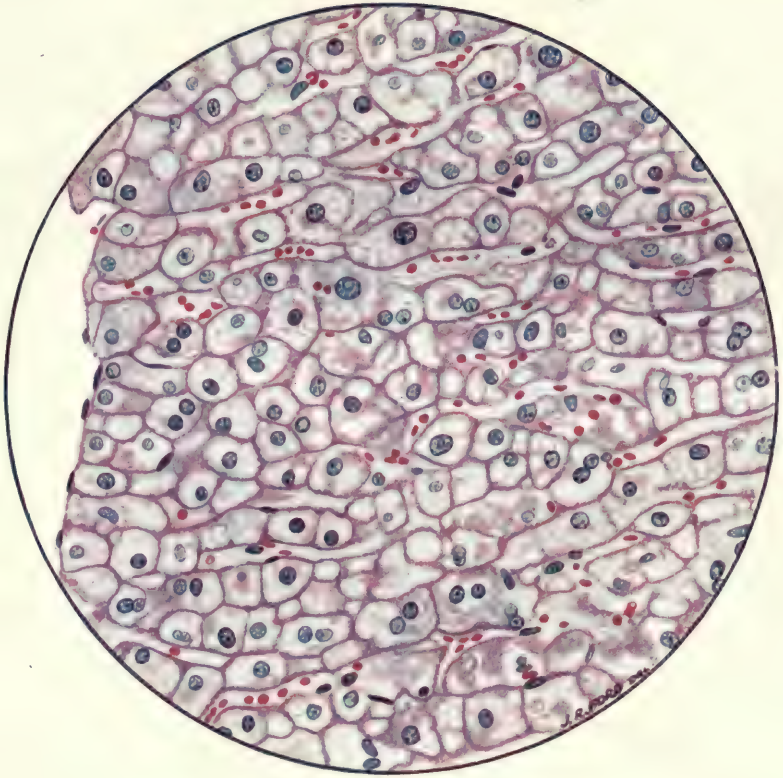

FIG. 1.—Liver of rabbit. Formaldehyde; paraffin; hæmatoxylin and eosin. × 335.

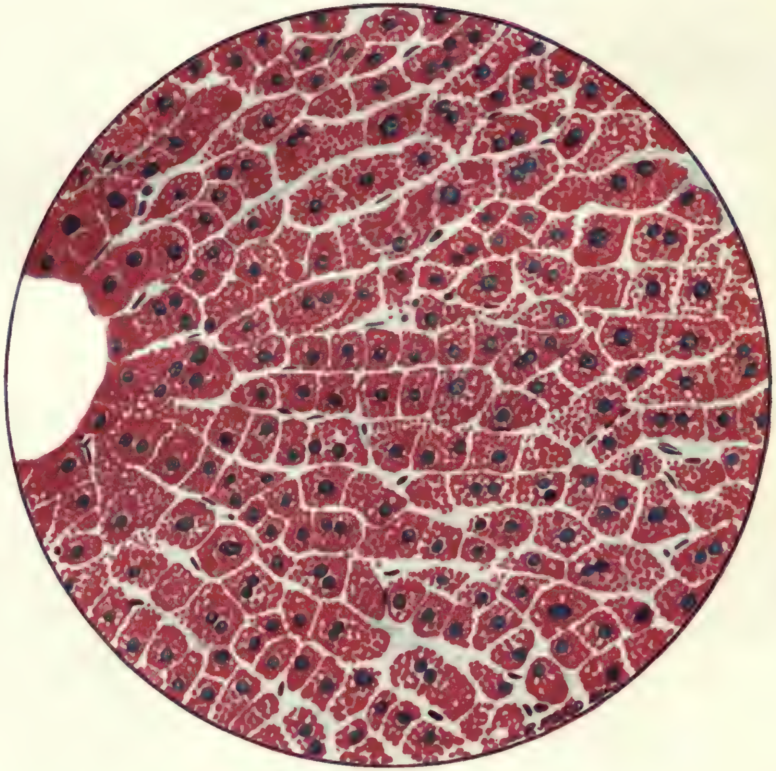

FIG. 2.—Same liver. Alcohol; celloidin; Best's glycogen stain. × 335.

Turnbull. Glycogenic Infiltration of the Liver of Laboratory Rabbits.

the central zones of the lobules. The nuclei showed great variation in size, in structure and in affinity for hæmatoxylin. In many cells no nucleus was present. Sir Frederick Andrewes, Prof. Bulloch, and my colleagues, Dr. G. B. Bartlett and Dr. W. W. Woods, kindly examined these sections and the sections of Groups 5 and 6. They all expressed the opinion that the changes were identical in nature.

It was impossible to doubt, therefore, that in the livers of the rabbits which had been inoculated with contaminated saline or with contaminated saline and salvarsan, the vesiculation and nuclear changes were expressions of a glycogenic infiltration such as can occur in a healthy rabbit after a suitable meal. It was impossible to regard these changes as evidence of experimentally induced degeneration.

The variations in the structure and staining affinities of the nuclei of the cells in which glycogenic infiltration was proved to be present were evidently expressions of physiological nuclear activity. Only a few types of these variations are included in the two fields selected for illustration. Some of the variations in the size of the nuclei and the complete absence of nuclei could be ascribed to thin sections of greatly swollen cells ; in a thin section a swollen cell might be cut external to its nucleus or through the periphery of its nucleus. The fact that absence of nuclei appeared to be more frequent in the thin paraffin sections than in the relatively thick frozen and celloidin sections confirms this. There were, however, cells without nuclei in thick sections. Further, in all sections there were small nuclei, frequently of irregular outline, so faintly stained as to be just recognisable. The appearances suggested, therefore, that karyolysis and complete loss of nuclei must be included among the changes which may occur during physiological activity of hepatic cells.

The single feeding experiment, described above, indicates a simple method of determining the phases through which the nucleus of the hepatic cell passes during glycogenic metabolism. To point out this line of research is not, however, the primary object of this paper. My object is to warn those who experiment upon animals against mistaking this physiological process for a pathological degeneration. Table I shows that in four out of seven experiments, in each of which two rabbits were employed, the estimated degree of vesiculation corresponded exactly in each pair of animals ; that is to say, in four out of seven pairs of animals the liver was in the same stage of physiological activity. In experiments such as these, in which one animal is employed as a control to the other, the stage of hepatic metabolism might well have corresponded in every pair of experiments ; the greater the care taken in selecting pairs of animals of like age and nutrition, and in ensuring that both animals were kept under exactly similar conditions, the more likely would this have been to occur. Had this occurred in the experiments under discussion, and had the vesiculation and nuclear changes been accepted as a pathological phenomenon, then the conclusion would have been inevitable that the contaminated water had caused degeneration of the liver, and that the addition of salvarsan to the water had not affected the result.

In conclusion, I thank the Salvarsan Committee for permitting me to publish this paper, and the Medical Research Council for defraying the expense of the reproduction of the beautifully executed paintings by Mr. J. R. Ford.

SUMMARY.

The histological changes seen in ordinary microscopic preparations of the rabbit's liver when infiltrated with glycogen after a suitable meal resemble closely those of a severe parenchymatous degeneration. The danger of mistaking in animal experiment these physiological changes for manifestations of degeneration is illustrated by an account of the findings in a series of experiments in which pairs of rabbits were inoculated respectively with contaminated water and with salvarsan dissolved in contaminated water. Incidentally attention is drawn to the promising field offered by glycogenic infiltration of the liver of the rabbit for the experimental study of changes in the nucleus during physiological activity of the hepatic cell.

INDEX TO VOL. I.

Actinomycosis, mycelial and other micro-organisms associated with human, 197
Adipose tissue, glandular, its relation to other endocrine organs, and to the vitamine problem, 184
Aërobic non-gas-forming bacteria, simple method of recording automatically the oxygen absorbed by, 66
Agglutinability of bacteria, variation in, associated with variation of cultural characters, 213
Anæsthesia, effect of surgical, on the reaction of the blood, 282
Antigenic properties of *B. psittacosis*, 131
Antigens, value of simultaneous testing for the Wassermann reaction with two different, 219

B. influenzæ, new medium for the growth of, 129
B. psittacosis, antigenic properties of, 131
Bacteria, aërobic non-gas-forming, simple method of recording automatically the oxygen absorbed by, 66
——— capsules of, technique for the demonstration of, 127
——— gas produced by, in culture, simple method of recording automatically, 66
——— influence of the reaction of media and the presence of buffer salts on the metabolism of, 288
——— variation in agglutinability of, associated with variation of cultural characters, 213
Bacteriological culture media, determination of the reaction of, 9
Baker, S. L., technique for the demonstration of the capsules of bacteria, 127
Bayliss, W. M., is hæmolysed blood toxic? 1
Bean, soy-, method for the estimation of urea by, 135
Benzidine peroxidase reaction, variations in the, 225
Blood, hæmolysed, toxicity of, 1
——— reaction of, effect of surgical anæsthesia on, 282
Blood, volume of, value of the vital-red method as a clinical means for the estimation of, 142
Buffer salts, influence of, on the metabolism of bacteria, 288

Cancerous and normal surviving mouse tissues, respiratory exchange of, 244
——— cells, comparative oxygen avidity of normal and, measured by their reducing powers on methylene-blue, 115
——— mouse tissues, oxygen consumption of normal and, *in vitro*, 175
Capsules of bacteria, technique for the demonstration of, 127
Classification, serological, of meningococci, 44
Clemenger, F. J., see *Fleming, A.*
Colebrook, L., the mycelial and other micro-organisms associated with human actinomycosis, 197
Collip, J. B., the effect of surgical anæsthesia on the reaction of the blood, 282
Colloidal gold test, value and mechanism of, 71
Cowan, Mary L., see *Maitland, H. B.*
Cramer, W., on glandular adipose tissue and its relation to other endocrine organs and to the vitamine problem, 184
——— on sympathetic fever and hyperpyrexial heat-stroke, 31
Cruickshank, J., the value and mechanism of the colloidal gold test, 71
Cultural characters, variation in agglutinability of bacteria associated with variation of, 213
Culture, gas produced by bacteria in, simple method of recording automatically, 66
——— media, see *Media.*

Dale, H. H., conditions which are conducive to the production of shock by histamine, 103
Detweiler, H. K., see *Maitland, H. B.*
Drew, A. H., the comparative oxygen avidity of normal and malignant cells measured by their reducing powers on methylene-blue, 115
Dysentery, researches on, 237

Encephalitis lethargica, experimental transmission of, in series in monkeys and rabbits, 257
——— ——— to a monkey, 89
——— spontaneous infection in a monkey, 257
Endocrine organs, their relation to glandular adipose tissue, 184
Epidemic influenza, see *Influenza.*

Fever, sympathetic, and hyperpyrexial heat-stroke, 31
FILDES, P., a new medium for the growth of *B. influenzæ*, 129
——— the serological classification of meningococci, 44
——— and MCINTOSH, J., the ætiology of influenza, 119, 159
Filter-passing virus, experiments in search of, in influenza, 263
FLEMING, A., and CLEMENGER, F. J., a simple method of recording automatically the gas produced by bacteria in culture and the oxygen absorbed by aërobic non-gas-forming bacteria, 66

Gas produced by bacteria in culture, simple method of recording automatically, 66
Glandular adipose tissue, its relation to other endocrine organs and to the vitamine problem, 184
Glycogenic infiltration of the liver of laboratory rabbits, 310
Gold test, value and mechanism of the colloidal, 71
GYE, W. E., see *Russell, B. R. G.*

Hæmolysed blood, toxicity of, 1
HARRIS, D. T., the value of the vital-red method as a clinical means for the estimation of the volume of the blood, 142
Heat-stroke, hyperpyrexial, sympathetic fever and, 31
Histamine, production of shock by, 103
Hyperpyrexial heat-stroke, sympathetic fever and, 31

"Ice-box" method, testing for the Wassermann reaction with the, 219
Influenza, ætiology of, 119, 159, 263
——— bacillus, new medium for the growth of, 129
——— experiments in search of a filter-passing virus in, 263

KENNAWAY, E. L., a method for the estimation of urea by soy-bean, 135

Lethargic encephalitis, see *Encephalitis lethargica.*
Litmus solution suitable for bacteriological purposes, 70
Liver of laboratory rabbits, glycogenic infiltration of, 310

MCINTOSH, J., a litmus solution suitable for bacteriological purposes, 70
——— transmission of experimental *encephalitis lethargica* in series in monkeys and rabbits, with notes on a spontaneous infection in a monkey, 257
——— and SMART, W. A. M., the determination of the reaction of bacteriological culture media, 9
——— and TURNBULL, H. M., the experimental transmission of *encephalitis lethargica* to a monkey, 89
——— see *Fildes, P.*
MACKIE, T. J., variation in agglutinability of bacteria associated with variation of cultural characters, 213
——— and ROWLAND, C. C., the value of simultaneous testing for the Wassermann reaction with two different antigens and the "ice-box" method, 219
MACLEAN, H., and WESSELOW, O. L. V. DE, on the testing of renal efficiency, with observations on the "urea coefficient," 53
MAITLAND, H. B., COWAN, MARY L., and DETWEILER, H. K., the ætiology of epidemic influenza: experiments in search of a filter-passing virus, 263
Malignant cells, see *Cancerous.*
Media, culture, determination of the reaction of, 9
——— ——— influence of the reaction of, on the metabolism of bacteria, 288
Medium, a new, for the growth of *B. influenzæ*, 129
Meningococci, serological classification of, 44
MENTEN, M. L., variations in the benzidine peroxidase reaction depending on fixative, physiological activity and type of animal, 225
Metabolism of bacteria, influence of the reaction of media and of the presence of buffer salts on, 288
Methylene-blue, comparative oxygen avidity of normal and malignant cells measured by their reducing powers on, 115
Micro-organisms, mycelial and other, associated with human actinomycosis, 197
Monkey, experimental transmission of *Encephalitis lethargica* to a, 89, 257
——— spontaneous infection of, with *Encephalitis lethargica*, 257
Mouse tissues, oxygen consumption of normal and cancerous, *in vitro*, 175
——— respiratory exchange of surviving, normal and neoplastic, 244
Mycelial and other micro-organisms associated with human actinomycosis, 197

Neoplastic, see *Cancerous.*

Oxygen absorbed by aërobic non-gas-forming bacteria, simple method of recording automatically, 66
——— avidity, comparative, of normal and malignant cells measured by their reducing powers on methylene-blue, 115
——— consumption of normal and cancerous mouse tissues *in vitro*, 175

Peroxidase reaction, variations in the benzidine, 225
PERRY, H. M., the antigenic properties of *B. psittacosis*, 131
Psittacosis bacillus, antigenic properties of, 131

Rabbits, laboratory, glycogenic infiltration of the liver of, 310
——— transmission of experimental *encephalitis lethargica* in series in, 257
Reaction, of the blood, effect of surgical anæsthesia on, 282
——— of bacteriological culture media, determination of, 9
——— of media, influence of, on the metabolism of bacteria, 288
Renal efficiency, testing of, 53
Respiratory exchange of surviving mouse tissues, 244
ROWLAND, C. C., see *Mackie, T. J.*
RUSSELL, B. R. G., and GYE, W. E., the oxygen consumption of normal and cancerous mouse tissues *in vitro*, 175
——— and WOGLOM, W. H., the respiratory exchange of surviving mouse tissues, normal and neoplastic, 244

Serological classification of meningococci, 44
Shock, production of, by histamine, 103
SMART, W. A. M., see *McIntosh, J.*
Soy-bean, method for the estimation of urea by, 135
Surgical anæsthesia, effect of, on the reaction of the blood, 282
Sympathetic fever and hyperpyrexial heatstroke, 31

TURNBULL, H. M., glycogenic infiltration of the liver of laboratory rabbits, 310
——— see *McIntosh, J.*
TWORT, F. W., researches on dysentery, 237

"Urea co-efficient," testing of renal efficiency, with observations on the, 53
Urea, a method for the estimation of, by soybean, 135

Vital-red method, value of, as a clinical means for the estimation of the volume of the blood, 142
Vitamine problem, glandular adipose tissue and its relation to other endocrine organs and to the, 184
Volume of the blood, value of the vital-red method as a clinical means for the estimation of, 142

Wassermann reaction, value of the "ice-box" method, 219
——— value of simultaneous testing for, with two different antigens, 219
WESSELOW, O. L. V. DE, see *MacLean, H.*
WOGLOM, W. H., see *Russell, B. R. G.*
WOLF, C. G. L., the influence of the reaction of media and of the presence of buffer salts on the metabolism of bacteria, 288

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